http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences, 2 (4): 1317-1321, 1999 Research Article

Induction of Mild Strains of Pepper Mottle Virus by Chemical Mutagenesis and Their Efficacy in Cross Protection

Athar Nadeem*, Zhongguo Xiong and Merritt Nelson,
Department of Plant Pathology, College of Agriculture, University of Arizona, Forbes 204,
PO Box 210036, Tucson, AZ 85721-0036, USA.
*Current address: Central Cotton Research Institute, Multan, Pakistan.

Abstract

The present study was conducted to test the effectiveness of nitrous acid induced mutants of PeMV in cross protection. Crude sap of pepper *Capsicum frutescens* L. 'NM 6-4' infected with pepper mottle virus (PeMV) was treated with nitrous acid to induce mutants. Twelve isolates were selected from two distinct types of local lesions produced by nitrous acid treatment on *Capsicum frutescens* L. 'tabasco'. Of the twelve isolates, five induced mild symptoms on both pepper foliage and fruit. When these mild isolates were inoculated to pepper plants and subsequently challenged with the wild type PeMV, they protected the plants from the severe effect of the wild type isolate. One hundred percent of marketable pepper fruit was produced from these protected plants.

Introduction

While genetic engineering represents the contemporary technology of choice for developing virus resistance (Gonsalves *et al.*, 1998) plant varieties based on a broad definition of cross protection, less sophisticated and more traditional methods are still functional and applicable to local conditions in some cases. Such traditional methods involve the use of mutagenic agents to produce mild strains for use in cross protection and may still be the best approach where potential economic benefits are localized and moderate. The principle is to treat virus with a mutagenic agent such as nitrous acid and select mild strains from the resulting mutations (Siegel, 1965, Yeh and Gonsalves, 1984; Yeh, *et al.*, 1988) Cross protection may be defined as the protection of a plant from infection by a severe virus by pre-inoculation with a mild strain of that virus (Lecoq, 1998). The cross protection was used to develop a regional management program for pepper mottle virus (PeMV) in southeast Arizona where PeMV is the most important virus present and the only known potyvirus (Nelson, and Wheeler, 1972; Nelson, and Wheeler 1978; Nelson, *et al.*, 1982). PeMV is also found in Florida, Mexico, California and New Mexico but generally in association with other potyvirus diseases (Nelson and Wheeler, 1972; Nelson and Wheeler 1978; Nelson, *et al.*, 1982; Zitter, 1972) that are similar in effects on peppers. The fact that PeMV is the only potyvirus present in the area this approach to management was an attractive one. The presence of other pepper potyviruses in an area would make such an approach more complicated.

Mild strains of viruses may be found in nature or can be induced by heat, or UV light in addition to chemical mutagens (Oshima, 1975; van Volten-Doting *et al.*, 1980; Kassanis and van Volten-Doting, 1982). Chemical mutagens, especially nitrous acid, have been proven to be most successful for RNA viruses. In the present study we test the effectiveness of nitrous acid induced mild mutants of PeMV in cross protection.

Materials and methods

Virus Isolates: Pepper mottle virus strain AZD (PeMV-AZD) was originally isolated from Cochise county, Arizona and was maintained in *Capsicum frutescens* L. 'New Mexico 6-4'. The virus produces local lesions on the leaves of *Capsicum frutescens* L. 'tabasco'. All pepper test plants and virus production plants were grown in 4-inch pots in a 50:50 peat sand mix. The tabasco plants were not used until they had developed woody stems. Such plants produced optimal lesions and were not vulnerable to stem necrosis and death as were younger plants (Nelson and Wheeler 1978).

Artificial Induction of PeMV-AZD Mutants: A powerful mutagen, nitrous acid (Gierer and Mundry, 1958; Muller and Costa, 1977; Siegel, 1965; Yeh and Gonsalves, 1984), was used to induce mutants from PeMV-AZD. Leaf tissue of pepper 'NM 6-4' infected with PeMV-AZD two to three weeks earlier was ground in distilled water (1 gm/4 ml). The crude sap was strained through cheesecloth and centrifuged in a Beckman JA-20 rotor at 8,000 RPM (7740xg) for 10 min. Aliquots of supernatant were divided into 4 x 1-ml parts. Sodium nitrite and sodium acetate (adjusted with acetic acid to pH 6.0) was added to a final concentration of 0.4 M and 0.1 M respectively. The reaction was stopped by adding an equal volume of 0.1 M phosphate buffer (pH 7.0) to 3 aliquots after 10, 20, and 30 minutes, respectively (T_1 = 10 min., T_2 = 20 min., and T_3 = 30 min.). In the control (T_0) treatment an equal volume of distilled water was added to bring the volume up to the levels of other treatments. All the mixtures were inoculated immediately to the local lesion host 'tabasco' pepper. Four plants were used in each treatment and 6

leaves per plant were inoculated. Local lesions appeared after 10-15 days and were recorded for each treatment. In further experiments, nitrous acid treatments were standardized at 25 minutes ($T_{2.5}$). It was anticipated that there would be a high degree of mutation in these treatments (Siegel, 1965). Single local lesions on 'tabasco' pepper leaves were removed with a sharp sterilized razor blade and ground in a few drops of 0.1 M phosphate buffer. Carborundum (22 μm, 600 mesh) was then added to the buffer. Young plants of 'NM 6-4' pepper, a systemic host, at 5-6 leaf stage were inoculated. Symptoms were recorded as they developed.

Testing of Mutants for Cross Protection Potential: Eleven mild mutant isolates were selected to test their effectiveness in cross protection. One severe isolate was included as control. Pepper ('NM 6-4') plants were grown in seedling trays and then transferred to 6 inch pots. Eight plants per isolate were used in the cross protection test. Tissues infected with the selected isolates were ground in 0.1 M phosphate buffer pH 7.0 at a ratio of 1 gm/2ml. Carborundum was added just before inoculation of the test plants using a plumbers' acid brush. All plants were randomly placed in greenhouse in separate blocks. Twenty days after inoculation, when symptoms had fully developed, half of the plants in each treatment were inoculated with the wild type isolate to test the ability of the mild mutants to cross protect against the wild type isolate. Peppers were harvested when almost all plants bore fruit and there were signs of reddening on fruit of some plants. Data on fruit marketability, weight, length and width were recorded. A scale of 1-4 was used to categorize the marketability of fruit. The descriptions of categories are as follow (Fig. 1)

1. Normal fruit, smooth surface, no chlorotic streaking to very few light green streaks,fruit straight to slightly curved
2. Smooth to slightly bumpy surface with few narrow chlorotic streaks, fruit slightly curved.
3. Surface bumpy, narrow chlorotic streaks with yellow color more pronounced, fruit slightly disfigured.
4. Surface very bumpy, numerous broad chlorotic streaks which tend to coalesce, fruit disfigured and generally smaller.

To avoid bias in evaluating the fruit marketability, the evaluator did not know the origin of the fruit during the evaluation. Plants were cut back and a reading on fruit quality was recorded for the second crop for all the isolates.

Results

Conditions for mutagenic treatment: Infectivity of PeMV-AZD infected plant sap decreased as time of nitrous acid treatment increased. Figure 2 shows the number of local lesions produced by PeMV treated with nitrous acid at different incubation times. The trend of the local lesion production on 'tabasco' was the same in two independent experiments. In experiment 1 number of local lesions decreased from 624 in control to 57, 21 and 1 at T1, T2, and T3 respectively. In experiment 2 the number decreased from 177 to 59, 11, and 1 at T1, T2 and T3 respectively. On the basis of these data, the time selected for nitrous acid exposure for mutant induction was 25 min. In this treatment, as most of the virus particles loose their infectivity, the survivors are more likely to be mutants (Siegel, 1965).

Selection of mutants from mutagenic treatment: Single lesion isolates were selected from four different mutation experiments on the basis of lesion morphology. Two types of local lesions were observed. Lesions of the first type were morphologically identical to the wild type but smaller in size. The wild type local lesions started as small necrotic spots and then continued to increase in size up to 15 days. Lesions of second type were chlorotic in the beginning but later developed a necrotic border. This type of local lesion was unique to the nitrous acid treated isolates. A total of 90 lesions (40 type 1 and 50 type 2) were selected and were transferred to 'NM 6-4' pepper. Symptoms on 'NM 6-4' pepper appeared 10-15 days after inoculation. Of special interest were those plants showing milder systemic symptoms. One of the isolates (7216) showed severe symptoms and unusual plant growth patterns (Figure 3). Twelve mild isolates and the severe isolate 7216 were selected for further studies. Effects of these mutants on pepper yields are summarized in Table 1. There were statistically significant differences among the isolates in the fruit weight, length, width, disease severity and percentage of marketable fruit. Disease severity and percentage of marketable fruit were used to categorize the 12 isolates into three groups. Isolates 3230, 7210 and 7216 produced severe symptoms on the fruit comparable to PeMV-AZD. Plants infected with isolates 3230, 7210 and 7216 produced the same quality of fruit (by weight) as the control but only 25, 12.5, and 50 percent respectively, as many marketable fruit as the healthy control. Isolates 4211, 7204, 7221, and 7224 produced milder symptoms on the fruit and most of the fruit was of marketable quality. Isolates 4214, 5301, 7201, 7205, and 7211 produced very mild symptoms and all of the fruit was of the marketable quality. The quality and weight of the fruit in the latter group was comparable to that from control plants.

Effectiveness of mutants in cross protection: Results from cross protection experiments are summarized in Table 1. In cross protection experiments, New Mexico 6-4 pepper plants were initially inoculated with the 12 described isolates as inducers. The infected plants were subsequently inoculated with the wild type PeMV as the challenge. Peppers were harvested at the end of the growing season. The end of the growing season was judged to have been reached when the oldest fruit began to show some "red". Data on percentage of marketable fruit, disease severity, fruit weight was collected to assess the effectiveness of cross protection by the mutant isolates against the wild

Table 1: Fruit gross weight and marketability of chili pepper *Capsicum frutescens* "NM 6-4' infected with mutants of pepper mottle virus, challenged and unchallenged with wild type.

	Unchallenged			Challenged		
Isolate	Weight	Severity	Percentage	Weight of fruits	Sevverity Category	Percentage
3230	165.13	3.1	25.0	171.49	2.5	52.0
4211	201.02	2.0	87.5	207.40	1.9	100.0
4214	215.01	1.4	100.0	197.08	1.3	100.0
5301	231.29	1.7	100.0	203.92	1.5	100.0
7201	185.57	1.4	100.0	180.80	1.3	100.0
7204	157.28	2.5	69.5	136.22	2.8	28.5
7205	179.19	1.8	100.0	176.33	1.7	87.5
7210	168.98	3.5	12.5	116.06	3.4	0.0
7211	119.88	1.0	100.0	159.01	1.0	100.0
7216	91.35	4.0	0.0	116.97	3.9	0.0
7221	176.40	2.3	50.0	169.07	2.3	58.0
7224	158.06	1.8	66.5	159.76	1.5	75.0
PeMV(wt)	168.21	3.4	0.0	165.29	3.0	25.0
Control	236.03	1.0	100.0	165.79	3.0	0.0

*Data is an average of two experiments. **1 = Normal; **4 – Severe stunting distortion of foliage and fruit

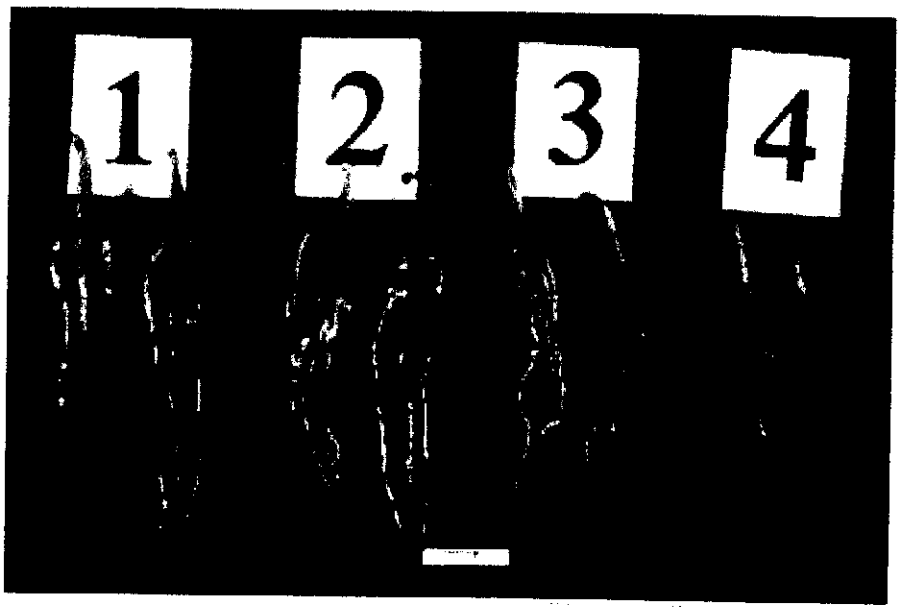

Fig. 1. Representative of different classes of fruits harvested from the cross protection experiment. This class scheme was used to categorize all the pepper fruits. Normal fruit, smooth surface, no chlorotic streaking to very few light green streaks, fruit straight to slightly curved. Smooth to slightly bumpy surface with few narrow chlorotic streaks, fruit slightly curved. Surface bumpy, narrow chlorotic streaks with yellow color more pronounced, fruit slightly disfigured. Surface very bumpy, numerous broad chlorotic streaks which tend to coalesce, fruit disfigured and generally smaller.

ype PeMV infection (Table 1). Out of 12 isolates tested, noculation with 5 isolates (4211, 4214, 5301, 7201 and 7211) apparently protected pepper from infection by the ild type PeMV. Plants initially inoculated with these olates produced good fruit weight and 100 percents rketable fruit after being challenged with the wild type MV. No severe symptoms associated with the wild type MV infection were observed in the challenged plants except the controls. Infection with another mild isolate (7205) did not protect infection by the wild type PeMV as well as others severe strains. This isolate induced mild symptoms by itself and infected plants yielded 100 percent marketable fruits. Percentage of marketable fruits dropped to 85 percent when plants inoculated with this isolate were challenged by the wild type PeMV. Plants protected by other isolates yielded good fruit weight but there was no

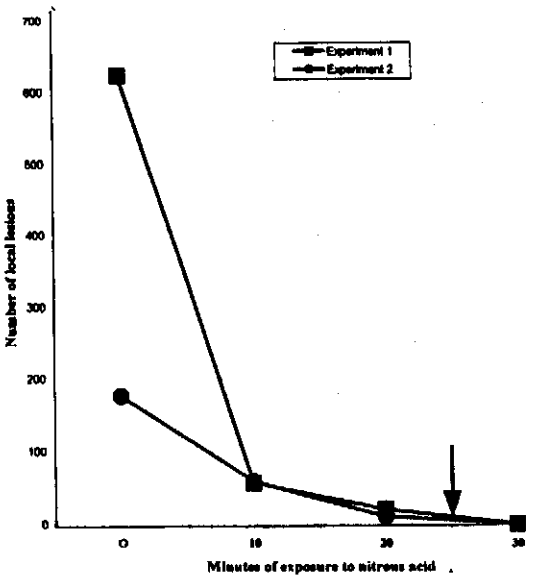

Fig. 2: Effect of nitrous acid treatment on number of pepper mottle virus (PeMV) local lesions on tabasco. Leaf extract of pepper *Capsicum frutescens* L. 'NM 6-4' infected with PeMV was treated with nitrous acid for different lengths of time and inoculated on *Capsicum frutescens* L 'tabasco' pepper. Numbers of local lesions produced were counted for each treatment. Figure illustrates the data from two separate experiments. In both experiments, number of local lesions decreased as the time to nitrous acid exposure increased. Arrow indicates the nitrous acid exposure time used in further experiments.

consistency for the quality in both challenged and unchallenged experiments. Isolates 3230, 7221, 7204, and 7210 offered no protection against the wild type isolate and quality of the fruits from the plants protected with these isolates was the same as that of the wild type infected plants.

Discussion

The goal of this work was to develop a mild strain of PeMV that could be used to protect peppers against infections by severe PeMV strains. Nitrous acid treatment was selected to induce mutations from the wild type PeMV. Two types of mutants were produced. One type of mutant produced abnormal chlorotic local lesions distinctly different from those of the wild type on 'tabasco' peppers. Of 90 local lesion isolates from 'tabasco' that were characterized, 50 originated from abnormal chlorotic local lesions. When attempts were made to transfer virus to a systemic host, most chlorotic lesions failed to show infectivity or produced isolates with either mild symptoms or no symptoms at all

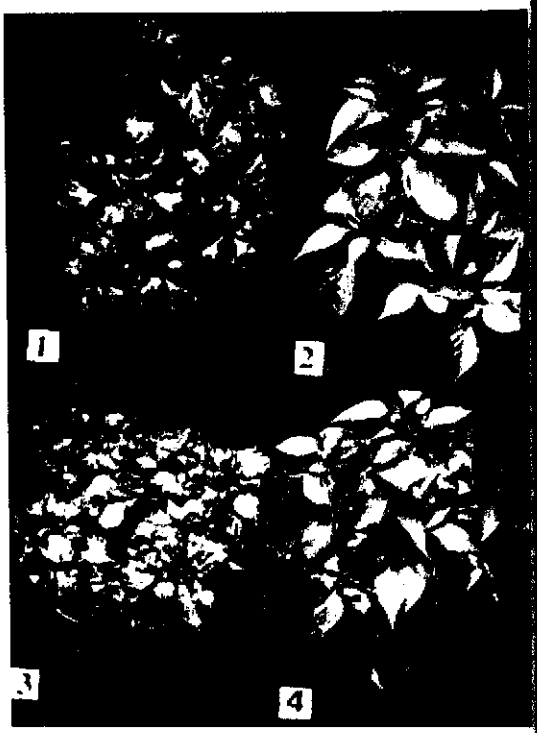

Fig. 3: Comparisons of the symptoms of the wild typ with two nitrous acid mutants of PeMV on pepp NM-64. Upper Left: Plant showing system mottle characteristic of the wild type PeM Upper Right. Plant infected with a mild isola 4214. Plant is healthy looking with very mi symptoms. Lower Left: Plant infected with isola 7216. The isolate produced very seve symptoms. Plant growth is more stunted compared with plant infected with the wild ty isolate: Lower Right: Healthy.

and gave poor reactions in ELISA tests. The properties these isolates did not meet the criteria for use as induc strains for cross protection. Two such isolates 3235 a 7203 were included in the trial but did not show a protection at all against the wild type PeMV. Two oth isolates of chlorotic origin (3230, 5301) which produc local lesions on 'tabasco' plants were also included in t trial. Isolate 3230 produced milder symptoms on leaves b severe symptoms on fruit with a reduced yield. Isola 5301 produced mild symptoms on fruit as well as plant a the yield was not affected. It appears that change in lesi morphology of the virus on local lesion host is not linked desirable characteristics useful in cross protection. In fa it is our opinion that these large abnormal 'tabasco' lesio represent defective mutants. Apparently lesion morpholo in this instant is not an indicator for mutation in the v genome, as evidenced by mild mutants selected from w type local lesions produced by virus treated with nitro acid. Most of the mild isolates tested, that originated fr

"normal" lesions on 'tabasco', gave some degree of protection from the wild type PeMV infection in challenge experiments. Other isolates from 'tabasco' lesions produced severe symptoms. Symptoms induced by these isolates were the same as or more severe than the wild type infection.

Of 12 isolates selected for detailed analysis, isolates 4214, 5301, 7201, and 7211 produced mild symptoms on both leaves and fruit in both challenged and unchallenged groups. These isolates were judged to be good candidates for use as inducers to protect against the challenger virus. This is a good example of traditional technology approach that can be used in specialty market situations. This type of approach has been successfully used to minimize losses due to severe infection of TMV and PRV (Gierer and Mundry, 1958; Siegel, 1965; Yeh and Gonsalves, 1984). Its application is simple requiring only standard laboratory, greenhouse equipment and supplies and no special training.

References

Gierer, A. and K. W. Mundry, 1958. Production of mutants of tobacco mosaic virus by chemical alteration of its ribonucleic acid. Nature, 183:1457-1458.

Gonsalves, D., S. Ferreria, R. Manschardt, M. Fitch, and J. Slightom, 1998. Transgenic virus resistant papaya: New hope for controlling papaya ringspot virus in Hawaii. APSnet feature September 1-30, 1998.

Lecoq, H., 1998. Control of plant virus diseases by cross protection. In Plant Virus Disease Control. Pp33-40. Eds. A Hadidi, R. K. Kheterpal, and H. Koganezawa. APS Press.

Muller, G. W. and A. S. Costa, 1977. Tristeza control in Brazil by preimmunization with mild strains. Proc. Int. Soc. Citriculture, 3: 868-872.

Nelson, M. R. and R. E. Wheeler, 1972. A new virus disease of peppers in Arizona. Plant Dis. Rep., 56:731-735.

Nelson, M. R. and R. E. Wheeler 1978. Biological and serological characterization and separation of potyviruses that infect peppers. Phytopath., 68:979-984.

Nelson, M. R., R. E. Wheeler and T. A. Zitter, 1982. Pepper mottle virus. No 253 in: Description of Plant Viruses. CMI/AAB. Kew, Surrey, England. 4pp.

Oshima, N., 1975. The control of tomato mosaic disease with attenuated virus of tomato strain of TMV. Rev. Plant. Prot. Res., 8: 126-135.

Roosien, J. and L. van Volten-Doting, 1982. Complementation and interference of ultraviolet induced mutants of alfalfa mosaic virus. J. Gen. Virol., 63: 189-198.

Siegel, A., 1965. Artificial production of mutants of tobacco mosaic virus. Adv. Virus. Res., 11: 25-60.

van Volten-Doting, L., J. A. Hasrat, E. Oosterwijk, P. van't Sant, M. A. Schoen and J. Roosien, 1980. Description and complementation analysis of 13 temperature sensitive mutants of alfalfa mosaic virus. J. Gen. Virol., 46: 415-426.

Yeh, S. D. and D. Gonsalves, 1984. Evaluation of induced mutants of papaya ringspot virus for control by cross protection. Phytopath., 74: 1086-1091.

Yeh, S. D., D. Gonsalves, H. L. Wang, R. Namba and R. J. Chiu, 1988. Control of papaya ringspot virus by cross protection. Plant Dis., 72: 357-380.

Zitter, T. A., 1972.Naturally occurring pepper virus strains in south Florida. Plant Dis. Rep., 56: 586-590.