VACCINE STRAIN OF TOMATO MOSAIC VIRUS OBTAINED AFTER SERIAL PASSAGES OF ATTENUATED STRAIN OF TOBACCO MOSAIC VIRUS IN TOLERANT TO TOBAMOVIRUSES TOMATO

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Summary

An attenuated strain of tobacco mosaic virus (TMV) isolated from tobacco and symptomless for tomato and pepper was put in fivefold passage in tolerant to tobamoviruses tomato. The changes in the strain population after the passages were studied by determination of the virion electrophoretic mobility, serological and biological properties of purified preparations of the obtained isolates. All performed experiments proved that as early as in the first passage TMV was replaced by a tomato mosaic virus (ToMV). After five serial passages the presence of virions causing mosaic in susceptible and/or tolerant tomato were not found. The study for vaccine properties of the new obtained isolate ToMV D52-V showed that it belonged to Pelham’s group “1” since it was effective in tomato carrying Tm-1 gene. Its protective action did not give way to that of analogous vaccines applied in the practice and hence strain D52-V could be successfully used for protection of susceptible and tolerant tomato against pathogenic tobamovirus strains.

Introduction

In the 70s and 80s of the twentieth century vaccine strains of tomato mosaic virus (ToMV) have been widely used for tomato protection against pathogenic tobamovirus strains. The application of this method in the practice proved its effectiveness however, some problems arose. The main objections were related to the presence of a little of pathogenic particles (up to 1 %) in the vaccine preparations and to the risk of appearance of new highly virulent virus strains. Apprehensions were stated about the vaccine strains translation upon other crops, for which these strains were pathogenic [7, 24]. The method application in tomato was restricted as consequence of the new cultivars creation - tolerant (Tm-1) or re-
istant to tobamoviruses ones (Tm-2 and Tm-2\(^2\)). However, the broad use of these cultivars led to raising of ToMV strains from group “1”, “2” and “1.2” according to Pelham’s classification [14], overcoming the respective resistance of the Tm-1, Tm-2 and Tm-1+Tm-2 genes [14, 15, 16, 20]. In tolerant to tobamoviruses tomato some “0” strains are able to cause symptomless systemic infection while “1” strains induce mosaic symptoms. Cultivars carrying Tm-1 gene are spread in our country and are used in the production of middle early and late tomato [20].

In Bulgaria, ToMV strains from groups “0” and “1” were found in yield tomato but strains of tobacco mosaic virus (TMV) were not isolated. The latter was occasionally established in green houses. However, in pepper TMV strains were frequently occurred besides ToMV ones and could even predominate [20]. The actual lack of TMV strains in tomato could be explained on one hand with the fact that TMV strains easily mutated in ToMV, as found in many investigations and on the other hand ToMV had a faster rate of multiplication or a greater invasiveness in tomato plants compared with TMV [26]. In the nature when TMV was found in tomato, it was mixed with ToMV [16, 20].

In the present work the obtaining of a new vaccine ToMV strain is described which could be used for protection of tomato with Tm-1 gene against pathogenic virus strains from group “1”.

Materials and Methods

Virus strains. Objects of the study were TMV strain D52 - symptomless for tomato and pepper and mild for tobacco as well as its derivative isolates obtained after passages in tolerant tomato.

Twenty one TMV and ToMV strains were included in the investigation. The ToMV vaccine strains were: the Bulgarian B-5 from group “0” after Pelham (National Bank for Industrial microorganisms and Cell Cultures 2024) and the Russian V-69 from group “1” (NBIMCC 2114) [21, 24]. The used pathogenic strains were: TMV - U1 (3405), TGM (3294), B (2022), MA (2077) and Sm (2125), the latter being from group “1”; ToMV strains from group “0” – ENP (2074), SD (2076), Lat-0 (2064), GdK (2075), GM-0 (2078), YM-0 (2348), No29 (3297), ToGM (3296); ToMV strains from group “1” – NH-69, SPS, GeBl (2112), So69 (2111), GM-1 (2349) and YM-1 (2348). The plants from which TMV and ToMV “0” strains have been isolated are shown in Table 2. The strains from group “1” originate from tomato. Strains TGM, B, GM-0, No29, ToGM, YM-0, GM-1 and YM-1 are from Bulgaria [19]. The authors express much gratitude to Prof. Dr. A. Th. B Rast, Prof. Dr. H. Laterrot and Prof. Dr. F. Garcia-Arenal for the supplying of other tobamoviruses.

Test plants. The following cultivars were used: tomato cv. Ideal susceptible to tobamoviruses and cv. Mobaci carrying Tm-1 tolerance gene to this virus group; tobacco cv. Samsun N’N’ (N’ gene from *Nicotiana sylvestris*) and cv. Samsun NN (N gene from *N. glutinosa*, localizing the tobamoviruses). The plants were grown in autoclaved soil and sterile metal pots. Seeds obtained from plants proved as virusless were used.

Virus propagation and purification. Tobacco plants cv. Samsun N’N’ in 5-6 leaf stage were inoculated with TMV strains. The multiplication of ToMV “0” strains was done in tomato cv. Ideal while strains from group “1” were propagated in tomato cv. Mobaci and plants in cotyledon-first real leaf stage were infected. The mosaic leaves were collected 21 days past inoculation (dpi) with pathogenic strains. The plants inoculated with vaccine
strains remained symptomless and all the leaves were harvested. Purified virus preparations were obtained by the method of Gooding and Hebert [5]. The virus concentration was spectrophotometrically determined. Tobacco cv. Samsun NN was used for production of single lesion isolates.

**Passages in tolerant tomato.** Tomato cv. Mobaci was inoculated with TMV D52 preparation (0.1 mg/ml), the plants were grown 45 days and the symptoms and the systemic virus distribution were recorded. All plants were symptomless and a purified preparation named D52-I was made from them. Tomato plants were again mass inoculated with this isolate and they remained symptomless. A preparation D52-II was similarly obtained. The procedure was multifold repeated and isolates D52-III, D52-IV and D52-V were purified. 40 plants were inoculated at every passage.

As in these experiments it was exceptionally important to exclude the contamination, besides the mentioned above measures all pots were enclosed with polyethylene. In this way the touch was avoided between the inoculated with different viruses plants, as well as the contact during watering.

**Electrophoretic mobility of virus particles.** The electrophoretic mobility (Rf) of tobamovirus virions was determined in 0.7 % agarose horizontal gel, in 40 mM Tris-acetate buffer, pH 7.8, containing 2 mM EDTA. The virus preparations were applied in concentration of 10 mg/ml. The electrophoresis was carried out at 60 V for 2.5 hours. The gels were stained with 0.2 % amido black in 0.1 M sodium acetate, containing 10 % glycerol [4]. The electrophoregrams were dried and stained additionally with Coomassie Brilliant Blue R-250 [27].

The Rf of the samples was accounted according to the equation Rf = S/F, where F was the distance from the start to the front and S was the sample distance. This value was calculated to Rf of the type strain U1 accepted as 100 %. The standard deviation of three repetitions was determined.

**Immunoelectrophoretic analysis.** Counter immunoelectrophoresis was performed in 0.9% agarose gel in 0.075 M veronal buffer, pH 8.6, at 150 V, 10 °C for two hours. The virus preparations were applied in concentration of 5 mg/ml (25 µg per start). The antiserum against ToMV in dilution of 1:4 was used. The gels were stained with Coomassie Brilliant Blue R-250 [27].

**Biological properties.** Ten plants of each tomato cvs Ideal, Mobaci and tobacco cv. Samsun N’N’ were inoculated with purified preparations of strain D52 and isolates D52-I, D52-II, D52-III, D52-IV and D52-V. The symptoms on the inoculated leaves were recorded on the 7th and 10th dpi and on the young leaves on the 40th day. The tobamovirus presence was proved by DAS-ELISA.

**Vaccine properties.** Two experiments for comparative testing of D52 and D52-V vaccine properties were carried out. In the first experiment, tomato plants cv. Ideal were vaccinated with B-5, V-69, D52 and D52-V and after 7 days they were challenge inoculated with TMV and ToMV pathogenic strains from group “0”. In the second case, tomato plants cv. Mobaci were vaccinated with B-5, V-69 and D52-V and after 7 days they were infected with group “1” pathogenic strains. The following variants were used as controls: untreated plants (healthy control); plants inoculated only with the respective vaccine strain and plants infected only with the respective pathogenic strain. The number of mosaic plants was accounted 30 days after the mosaic symptoms appearance in the plants infected only with pathogenic strains. 40 plants were used in each variant.
Results and Discussion

TMV strain No. 52 was isolated when the tobamovirus strain variability in commercial tobacco in Bulgaria was studied. This strain does not differ in electrophoretic mobility and serological properties from the typical TMV strains but it induces mild green mosaic in tomato, pepper and tobacco [17, 19]. A significant percentage (12 %) symptomless plants was found after single lesion infection of tomato (Tm+) with No. 52. After a purposive selection a TMV strain D52 was obtained that was symptomless for tomato and pepper and mild for tobacco. In spite of the multifold passages of strain D52 in tomato (Tm+), ToMV virions were not established in its preparations (testing on tobacco cv. Samsun N’N’).

Passages and biological properties of the obtained tobamovirus isolates

Five consecutive passages of strain D52 in tolerant to tobamoviruses tomato (Tm-1) was performed. The initial infection was made with a preparation obtained from tobacco cv. Samsun N’N’ in order to exclude the opportunity of ToMV particles presence in the started inoculum.

All the tomato plants remained symptomless during a period of 45 days although the virus presence in them was proved by DAS-ELISA. Preparations of isolates D52-I, D52-II, D52-III, D52-IV and D52-V were purified. When studying the biological properties of the isolates it was found that they produced necrotic local lesions on the Samsun N’N’ inoculated leaves without systemic infection. On the contrary, TMV strain D52 infected systemically this cultivar and did not cause necrotic local lesions. Therefore, as early as at the first passage TMV D52 was replaced by ToMV. However, the five consecutive passages did not induce other changes in the isolates biological properties (Table 1). The only visible result was gradually increasing of the virus yield in the respective isolates. The D52-V yield obtained from tomato cv. Mobaci (Tm-1) was approximately equal to that of D52 from tomato cv. Ideal (Tm+).

Table 1. Symptoms* induced by strain D52 and its isolates obtained after passages in tolerant to tobamoviruses tomato (Tm-1).

<table>
<thead>
<tr>
<th>Strain/isolate</th>
<th>Tomato cv. Ideal (Tm+)</th>
<th>Tomato cv. Mobaci (Tm-1)</th>
<th>Tobacco cv. Samsun N’N’</th>
<th>Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>D52</td>
<td>SL</td>
<td>SL</td>
<td>MGM</td>
<td>TMV</td>
</tr>
<tr>
<td>D52-I</td>
<td>SL</td>
<td>SL</td>
<td>NLL</td>
<td>ToMV</td>
</tr>
<tr>
<td>D52-II</td>
<td>SL</td>
<td>SL</td>
<td>NLL</td>
<td>ToMV</td>
</tr>
<tr>
<td>D52-III</td>
<td>SL</td>
<td>SL</td>
<td>NLL</td>
<td>ToMV</td>
</tr>
<tr>
<td>D52-IV</td>
<td>SL</td>
<td>SL</td>
<td>NLL</td>
<td>ToMV</td>
</tr>
<tr>
<td>D52-V</td>
<td>SL</td>
<td>SL</td>
<td>NLL</td>
<td>ToMV</td>
</tr>
</tbody>
</table>

*Symptoms: mild green mosaic (MGM), necrotic local lesions (NLL), symptomless (SL).
Electrophoretic mobility of the new obtained isolates

It is known that TMV strains differ significantly in their electrophoretic mobility from ToMV ones [8, 23, 29] and this characteristic could be used for distinguishing both viruses [17]. The strain D52 Rf was determined as well as the Rf of its new obtained isolates. Strains U1 for TMV, GM-0 and GM-1, respectively for ToMV groups “0” and “1”, were used as control viruses (Fig. 1). It could be seen that there was not statistically proved difference between the mobility of ToMV “0” and “1” stains but this difference was significant between TMV and ToMV. The D52 Rf was equal to that of U1 and all isolates obtained after the passages moved as ToMV strains in the gel.

The electrophoretic pattern of the initial strain and the control ones as well as the lines of some obtained after passages isolates are shown in Fig. 2.

![Fig. 1. Electrophoretic mobility of strain TMV 52 and the isolates obtained after serial passages in tolerant tomato. Control strains: for TMV – U1, for ToMV group “0” – GM-0 and ToMV group “1” – GM-1. Data are the means of three replications. The standard deviation is presented.](image1)

![Fig. 2. Electrophoretic analysis of virus particles of strain TMV 52 and the isolates obtained after serial passages in tolerant tomato. Control strains: for TMV - U1, for ToMV group "0" - GM-0 and ToMV group "1" - GM-1.](image2)

Lines:
1. TMV U1
2. TMV 52
3. Isolate 52-I
4. Isolate 52-V
5. ToMV GM-0
6. ToMV GM-1
Serological properties of the new obtained isolates

The change in the serological relationship of the obtained after passages in tolerant tomato isolates and the initial TMV strain D52 was followed (Fig. 3). The precipitation line of the preparation obtained after the first passage formed spur towards TMV D52. The same was the result for the isolates after the next passages. On the other hand, D52-I, D52-II, D52-III, D52-IV and D52-V reacted as serologically identical to the control ToMV strain GM-1. A fusion of the precipitation lines was observed. This data shows a change of TMV D52 in ToMV as early as at the first passage in tomato with Tm-1 gene.

A method for group determination of symptomless strains by screening pathogenic revertants in the strain populations and their classification according to Pelham was proposed by Suchov et al. [24]. The authors applied this approach for group determination of the vaccine V-69, in whose population about 1% pathogenic virus particles from group “1” was proved. The presence only of “1” pathogenic particles indicates that the vaccine strain is from the same group. 250 tomato plants cv. Ideal were single lesion inoculated with isolate D52-V and all of them remained symptomless. This result required searching of new way for the pathotype determination of the isolate.

Vaccine properties of strain D52 and isolate D52-V

The effectiveness of the protective action of a given vaccine strain depends mainly on two factors. The first one is availability of a good adaptation of the strain to the plants that it protects. Usually the vaccine strains are searched in the virus populations spread in the respective crop [24] or, if they have been isolated from other culture, the multifold consecutive passages in the plants, for which a vaccine aims to be obtained, improve these properties [20]. ToMV strains that have originated from tomato and pepper and possess good vaccine effect to the respective culture can not protect the tobacco against pathogenic TMV and ToMV strains [12, 18]. The second factor is the relationship degree between the vaccine and the pathogenic strains. As their relationship is closer so the interference between them is more strongly expresses i.e. the vaccine effectiveness against these strains is higher [16, 18].

On this basis we supposed that the pathotype of the isolates obtained after passages of strain D52 could be determined comparing their protective properties with ones of other known vaccine from groups “0” and “1”. At the same time, as D52 and D52-V are symptomless for tomato and pathogenic virions were not found in their preparations, it was interesting to study their protective activity.

The results obtained after comparing the vaccine properties of D52 and D52-V with two
well known vaccines B-5 and V-69 in tomato cv. Ideal are presented in Table 2. A susceptible to tobamoviruses tomato cultivar was used because strain D52 could not infect tolerant tomato (Tm-1). All challenge strains were from group “0”. They are isolated from different cultures of family Solanaceae and belong to TMV or ToMV.

Table 2. Vaccine properties of B-5, V-69, D-52 and D52-V in tomato cv. Ideal (Tm+) against pathogenic tobamovirus strains.

<table>
<thead>
<tr>
<th>Pathogenic strains</th>
<th>Vaccine strains</th>
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<tbody>
<tr>
<td>Viruses</td>
<td>Strains</td>
</tr>
<tr>
<td>TMV</td>
<td>U1</td>
</tr>
<tr>
<td>&quot;</td>
<td>TGM</td>
</tr>
<tr>
<td>&quot;</td>
<td>B</td>
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<tr>
<td>&quot;</td>
<td>MA</td>
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<tr>
<td>ToMV</td>
<td>ToGM</td>
</tr>
<tr>
<td>&quot;</td>
<td>No29</td>
</tr>
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<td>&quot;</td>
<td>GM-0</td>
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<td>&quot;</td>
<td>ENP</td>
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<td>&quot;</td>
<td>SD</td>
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<td>&quot;</td>
<td>YM-0</td>
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<td>&quot;</td>
<td>Lat-0</td>
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<td>&quot;</td>
<td>GdK</td>
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</table>

*Number of symptomless plants / total number of tested plants x 100 (%).

The plants infected only with the one of the fourth vaccine as well as the control untreated plants remained symptomless to the experiment end. Up to 15th dpi all plants infected only with pathogenic strains developed mosaic symptoms. The percentage of symptomless plants inoculated initially with vaccine and after that with pathogenic strain varied in quite wide range. Only single mosaic plants were observed in a part of the variants where the protective properties of both vaccines and D52-V were tested. In analogous variants of TMV D52, the lowest portion of symptomless plants was found.

D52-V had very good vaccine activity and the symptomless plants percentage varied from 85 to 100 %. In variants when the challenge strains were TMV, D52-V showed better vaccine properties than the others, with exception of the variant B-5+U1 where it was smaller and of the variant V-69+B where the values were equal. There were least symptomless plants in the combination D-52-V+No.29, in which the pathogenic strain was TMV isolated from pepper.

From the obtained results it could be concluded that D52 and D52-V protected tomato against pathogenic TMV and ToMV strains originated from different cultures, as TMV D52 gave way to ToMV strains V-69 and B-5 in vaccine properties while ToMV D52-V
equalized with them. This fact completely correlates with the literature data that the effectiveness of the protection depends on whether the strain is isolated from the plant species it have to protect, which presume also a good adaptation to it [9, 12, 18].

The “0” strains propagate difficult in tolerant to tobamoviruses tomato and accumulate in low concentrations, and in some cases they can not multiply in them at all [22, 28]. Hence, these viruses could not possess protective properties against pathogenic “1” strains in tolerant tomato. In order to be proved that D52-V is from group “1”, its vaccine action was compared with that of strain V-69 which was from the same group as well as with strain B-5 that multiplied systemically in tolerant tomato but was from group “0” [24]. The experiment was carried out with the tolerant tomato cv. Mobaci and pathogenic “1” strains were only used (Fig. 4). The percentage of the symptomless plants was 92.5-100 % for V-69 and 95-100 % for D52-V. The differences were small and the deviations were in favor to the one as well as to the second vaccine. In the same time, only single symptomless plants were established in the variant vaccinated with B-5 although the strain exhibited excellent protective effect in susceptible tomato cultivars.

These results allow considering that isolate D52-V is from group “1” and could be successfully applied as vaccine in susceptible and tolerant to ToMV tomato.

![Graph](image)

Fig. 4. Vaccine properties of V-69 and 52-V in tolerant to tobamoviruses tomato cv. Mobaci (Tm-1). The vaccine strain B-5 from group “0” is presented for comparison.

It is known that the capsid protein is responsible for the appearance of hypersensitive reaction in tobacco with N’ gene resulting in death of the infected cells [1, 2, 3, 11, 25]. Consequently, it could be supposed that all isolates obtained after the passages in tolerant tomato consist of virions, in which there is mutation(s) in the capsid protein. It simultaneously leads to changes in the Rf and serological properties of the virus as well as to necrotic local lesion appearance on the inoculated leaf of tobacco with N’ gene.

The ability of normal virus propagation in tomato with tolerance Tm-1 gene is connected with amino acid substitutions in the RNA-dependent RNA polymerases [6, 13, 28]. Probably isolate D52-V consists of virions possessing the respective mutation as it is from group “1”. It is established that attenuated and protective properties of ToMV strains are related with mutations in the virus RNA encoding the same proteins [10].
Therefore, it could be assumed that D52 and D52-V distinguish from the typical pathogenic strains from groups "0" and '1" in amino acid alterations again in the RNA-dependent RNA polymerases. The occurred changes in the respective genes as a result of the passages in tolerant tomato do not concern these regions coding for attenuated properties of D52. The replacing of TMV D52 with ToMV isolate D52-I after the first passage and the subsequent mutations selection, resulting in the multiplication adaptation of the virus population in tolerant tomato, leads also in improving the vaccine properties of D52-V in comparison with D52.

The obtained isolate D52-V protecting excellent susceptible and tolerant tomato against pathogenic tobamovirus strains is very stable because pathogenic virions are not found in its preparations. In this characteristic it exceeds known and widely used in practice vaccines [16, 24]. At the same time, it is symptomless for pepper, a culture often growing in adjacency of tomato. That is why, the vaccinated with D52-V tomato could not be virus source for tobacco mosaic disease in pepper cultivars susceptible to tobamoviruses. Consequently, the objections against using vaccine strains do not referred to D52-V that makes it appropriate for application in the production of green house and field tomato.

References

ВАКСИНЕТ ТоМВ ЩАМ, ПОЛУЧЕН ЧРЕЗ ПОСЛЕДОВАТЕЛНИ ПАСАЖИ НА АТЕНЮИРАН ТМВ ЩАМ В ТОЛЕРАНТНИ НА ТОБАМОВИРУСИ ДОМАТИ

Елисавета Стоименова*, Анжела Йорданова

Резюме

Атенюиран щам на тютюновомозаичния вирус (TMV), изолиран от тютюн и безсимптомен за домати и пипер, е подложен на петкратни пасажи в толерантни на тобамовируси домати. Промените в популацията на щама след съответните пасажи е изследвана чрез определяне на електрофоретичната подвижност, серологичните и биологичните свойства на пречистени препарати на получените изолати. Опитите доказват, че още след първия пасаж TMV се измества от вируса на доматовата мозайка (ToMV). След пет последователни пасажи щама не е установено наличието на вириони, предизвикващи мозайка в чувствителни и/или толерантни домати. Проучването за ваксинни свойства на новополучения ToMV изолат D52-V показва, че той принадлежи към "1" група по Пелхам, т.е. е ефективен при домати с ген Tm-1. Неговото защитно действие не отстъпва на това на аналогични ваксини, прилагани в практиката и следователно щам D52-V може успешно да се използва за защита на чувствителни и толерантни домати от патогенни щамове на тобамовирусите.