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Begomovirus susceptibility in wild Nicaraguan tomato populations

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Begomovirusmottaglighet i vilda nicaraguanska tomatpopulationer

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Sammanfattning

En vanlig och viktig jordbruksgröda i Nicaragua är tomat. Under de senaste årtiondena har många tomatskördar i landet gått förlorade på grund av begomovirus, som tillhör familjen *Geminiviridae*. Begomovirusgenomet består oftast av två cirkulära komponenter av enkelsträngat DNA: DNA-A och DNA-B. Vissa begomovirus har enbart en genomkomponent, t.ex. *Tomato yellow leaf curl virus* (TYLCV). Ett sätt att förhindra en begomovirus-epidemi är att förädla fram en tomatsort med resistens mot begomovirus, då används ofta genotyper från vilda tomater. Syftet med detta projekt var att screena 20 vilda tomatpopulationer efter en population som uppvisar tecken på resistens. Dessutom undersöktes huruvida de vilda tomatpopulationerna hade blandade infektioner av olika begomovirus, en drivande kraft i evolutionen av begomovirus. Polymeras-kedjereaktion (PCR) med det generella primerparet AV494 and AC1048 användes för att detektera begomovirus i tomatproven. För att undersöka huruvida proven hade blandade infektioner kördes rolling circle amplification (RCA) på proven som sedan klyvdes med restriktionsenzymen *Sal*I, *Hpa*II och *Eco*RI. Inga blandade infektioner upptäcktes i de vilda tomatpopulationerna. I några av proven detekterades bara en begomoviruskomponent. Alla undersökta tomatplantor visade sig vara mottagliga för begomovirusinfektion. En av populationerna blev dock inte lika lätt infekterad och uppvisade relativt milda symtom efter infektion. För att fastställa om denna genotyp skulle kunna användas i förädlingssyfte krävs vetskap om den bär gener som tidigare associerats med begomovirusresistens, eller möjligtvis en tidigare okänd resistensgen. Dessutom bör det fastställas mot vilka begomovirusarter genotypen möjligtvis är resistent.

Abstract

Tomato production is a common and important agricultural activity in Nicaragua. During the last decades tomato producers have suffered great yield losses due to begomoviruses (family *Geminiviridae*). The genome of begomoviruses generally consists of two circular ssDNA components, DNA-A and DNA-B. *Tomato yellow leaf curl virus* (TYLCV) is an example of a begomovirus with only one component. An approach to manage the virus epidemics has been to breed a cultivar with begomovirus resistance genes, which have been transferred from wild tomato. The objective of this project was to investigate the begomovirus susceptibility of 20 wild tomato populations, with the aim to find a population with resistance. As an additional aim the presence of mixed begomovirus infections in the plants was investigated. Mixed infections are a driving force in begomovirus evolution. In order to detect begomoviruses in the tomato samples polymerase chain reaction (PCR) was run, using the degenerate primer pair AV494 and AC1048. In order to determine the presence of mixed infections in the samples rolling circle amplification (RCA) was carried out. Subsequently, the amplified DNA was digested by the restriction enzymes *Sal*I, *Hpa*II and *Eco*RI in order to determine the number of begomovirus components in each sample. No mixed infections were detected in the plants. In some of the investigated samples only one begomovirus component was detected. All wild tomato plants were found to be susceptible to begomovirus infections. However, one of the populations was not as easily infected as the other, and showed relatively mild symptoms after infection. In order to determine if this genotype could be used for breeding purposes, it is necessary to investigate if it has any of the genes previously found to be associated with begomovirus resistance, or a previously unknown resistance gene. Moreover, if resistance is detected it is essential to determine to which begomovirus species the genotype is resistant.

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1. Introduction

Agriculture is of great importance in Nicaragua. As in many other developing countries a majority of the Nicaraguan population depends on small-scale farming for food and income. The traditional exporting crops coffee and cotton have decreased during the last decades, partly due to low world market prices. During the 1980´s non-traditional crops like tomato (*Solanum lycopersicum*), pepper and melon were introduced in Nicaragua in order to increase export. Today these crops are among the most common components of the crop system used by farmers all over the country, and are grown during the rainy season (May-December) as well as during the dry season (January-April) under irrigation. However, the major consumption crops in Nicaragua are maize and bean, whereas tomato, pepper and cucurbits are the most common cash crops (Rojas, 2004).

Begomoviruses (genus *Begomovirus*, family *Geminiviridae*) are among the most destructive plant viruses in sub-tropical and tropical regions. Begomoviruses are transmitted by the whitefly *Bemisia tabaci,* which attacks a wide range of plants, e.g., bean, tomato and pepper. Thus, the distribution of begomovirus in a region is directly associated with the population dynamics and pressure of *B. tabaci* (Morales et al., 2006). The circular genome of begomoviruses is most often bipartite and consists of single stranded DNA. *Tomato yellow leaf curl virus* (TYLCV) is an example of a monopartite begomovirus. The genome components, DNA-A and DNA-B, have a size of approximately 2.5-2.8 kb each. For bipartite viruses, both components are essential for infection (Seal et al., 2006). However, different concentrations of the two components can be present in infected tissue. In this case, DNA-A is the component present in higher concentration (Inoue-Nagata et al., 2004). A begomovirus infection can seriously affect plant photosynthesis and disrupt flower and fruit formation. Typical infection symptoms are mosaic, yellowing, downward curling and distortion of the leaves, as well as stunting of the plant. Early infection often results in total crop loss (Rojas, 2004). However, some tomato plants do not become seriously affected by a begomovirus infection, and they are tolerant or resistant. Resistance means that the host plant carries genes that enables it to suppress virus multiplication and therefore also the development of disease symptoms. Tolerance is the instance when the host expresses mild symptoms after an infection, but supports normal levels of virus multiplication (Lapidot & Friedmann, 2002).

During the intense tomato production in Nicaragua in the 1980´s, begomovirus infections appeared and the virus was detected for the first time in 1986 (Rojas, 2004). The number of begomoviruses capable of infecting tomato increased during this period due to more and new hosts for the vector *B. tabaci,* increased use of pesticides that led to resistant whitefly populations and the lack of technical support for the farmers. These factors led to the severe begomovirus problem that had its peak in the 1990´s with enormous tomato yield losses and subsequent economic losses as a result (Rojas, 2004; Morales et al., 2006). Successful management of begomovirus epidemics, such as cropping system approaches, has been rare (Seal et al., 2006). Therefore, it has been determined that begomovirus-resistant tomato varieties are necessary for controlling the epidemics (Rojas, 2004; Morales et al., 2006). Ever since the begomovirus problem emerged, efforts to breed cultivars resistant to the viruses have been made. Several cultivars resistant to one of the most destructive begomoviruses, TYLCV, have been developed. The breeding strategy has been to screen wild tomato plants for resistance. The cross of the identified resistance into tomato cultivars is difficult since the resistance genetics is complex and often controlled by multiple genes (Lapidot & Friedmann, 2002). Another factor that complicates begomovirus management is recombination. Recombination is a driving force in begomovirus evolution and can lead to new and more

virulent strains or even new begomovirus species. Mixed infection, i.e., when more than one virus is infecting a plant, supplies the condition for recombination (Ala-Poikela et al., 2005). However, several hybrids carrying resistance have been developed and used commercially (Lapidot & Friedmann, 2002).

Recently, a project was established that involves a number of countries in Central America, and that is working towards improving the situation for tomato producers in the region. The project aims to find begomovirus tolerance genes in wild tomato species, which are collected in the countries of the consortium. Furthermore, the collected material will constitute the basis for a tomato DNA-bank, in order to establish a breeding program in Central America (Fontagro project plan).

The objective of this bachelor project was to investigate the susceptibility of wild Nicaraguan tomato plants to begomoviruses. If possible resistance/tolerance is detected, further investigation of the tomato DNA can be carried out, and the material can possibly be incorporated into a national breeding program. Furthermore, as an additional aim, the presence of mixed infections in the tomato plants was investigated. The objectives of the project can be summarized in the following questions:

- *Are the wild tomato plants susceptible to begomovirus infection? Do any of the populations show sign of begomovirus tolerance/resistance?*
- *Do the wild tomato plants have mixed begomovirus infections?*

2. Materials and Methods

2.1 Description of wild tomato plants

Tomato plants from 20 different wild tomato populations had previously been collected from the following areas around Nicaragua: Estelí, Matagalpa, Jinotega, Nueva Segovia, RAAS (Caribbean Coast) and Chinandega (**Table 1** and **Fig. 1**). The wild tomato plants were found growing in ditches, gardens or close to farmers` fields and were identified as wild due to phenotypic characteristics, such as small fruit size. The collected tomato plants did not show any signs of begomovirus infection. From each population, 3-4 plants were gathered, and each population was subsequently multiplied through self-pollination to approximately 30 plants.
Plants from two of the 20 populations showed different phenotypic characteristics (different stem color) within the population, namely the populations with number 11 and number 13. These two populations were subsequently divided into 11.1/11.2 and 13.1/13.2. (Personal communication: Tomas Laguna 2010-05-27)

Seeds from the wild plants were planted in a greenhouse on the 26th of March 2010 at the INTA (Instituto Nicaraguense de Tecnología Agropecuaria) experimental center in Sebaco, Nicaragua. After 33 days (on the 28th of April) the tomatoes were moved to the field. All tomato plants were handled in the same manner, being treated with fertilizers and pesticides at two occasions. To which species the different populations belong has not yet been determined (Personal communication: Tomas Laguna 2010-05-27).

Table 1. Location of the collection sites where the wild tomato plants from the 20 populations investigated in this project were gathered.

Tomato population	Collection site
1	Estelí
2	Estelí
3	Matagalpa
4	Matagalpa
5	Jinotega
6	Estelí
7	Estelí
8	Nueva Segovia
9	Nueva Segovia
10	RAAS (Caribbean Cost)
11.1	Matagalpa
11.2	Matagalpa
12	Jinotega
13.1	Chinandega
13.2	Chinandega
14	Chinandega
15	Chinandega
16	Chinandega
17	Chinandega
18	Chinandega
19	Chinandega
20	Estelí

Fig. 1. Map of Nicaragua. The collection sites where the 20 wild tomato populations were gathered are marked. Estelí (A), Matagalpa (B), Jinotega (C), Nueva Segovia (D), RAAS (E) and Chinandega (F).

2.2 Collection of samples

During the beginning of the rainy season tomato samples were collected at three different occasions in Sebaco. At each occasion, one plant sample was collected from each tomato population. However, two samples from population 11 were collected at each occasion, one sample from 11.1 and one sample from 11.2. The same procedure was applied for population 13. At the first occasion, 20th of April, the plants grew in a greenhouse and were approximately three weeks old. The tomato plants showed no signs of begomovirus infection. Leaf samples from one plant from each of the populations were put in plastic bags and stored at - 20° C until analysis.

On the 13th of May the second sampling occasion took place. The tomato plants had at this point grown in the field for approximately two weeks. The overall status of the plants was good. Some few plants in populations 6, 8, 10, 11.1, 14 and 18 showed mild signs of begomovirus infection, such as yellowing and curling (**Fig. 2**). In addition, the whitefly *B. tabaci* was observed in and around the tomato field. Young top leaves from one plant from each of the populations were pressed onto FTA-cards (Whatman); if possible leaves from a plant with symptoms were chosen. The FTA-cards were stored at room temperature until analysis.

The third sampling occasion took place on the 27th of May. The plants were now two months old and had grown in the field for one month. The majority of the tomato plants showed mild symptoms of begomovirus infections. Young tomato leaves from one plant with infection symptoms from each tomato population were put in a plastic bag and stored at - 20° C until analysis. However, plants of population 11.1 did not have any explicit begomovirus symptoms.

Fig 2. Putative begomovirus symptoms on a tomato plant from the second sampling occasion. The plant on the picture shows symptoms that could be derived from a begomovirus infection, i.e. yellowing and curling of the leaves.

2.3 Polymerase chain reaction

In order to detect begomovirus in the tomato samples, DNA from the tomato tissue was extracted according to the direct PCR-based detection of begomovirus, originally described by Wyatt & Brown (1996). DNA from the FTA-cards was extracted using the Extract-n-AmpTM Plant PCR kit (Sigma) essentially as described by Shepherd et al. (2008). A piece of the card with the size of a PCR-tube lid was placed in a PCR-tube. Fifty µl of extraction buffer was added to the PCR-tube, which then was vortexed vigorously. The tube was subsequently incubated at 95° C for 10 minutes. Fifty µl of dilution buffer was added and the tube was again vortexed vigorously. The extract was stored at - 20° C until PCR. As negative controls, PCR was run without extracted DNA.

PCR was run on all samples from the first (20th of April), and third (27th of May) sampling occasion. From the second sampling occasion (13th of May), PCR was run on samples showing symptoms of begomovirus infection. The degenerate primer pair AV494 and AC1048 (Wyatt & Brown, 1996) was used in order to amplify a highly conserved region of the begomovirus coat protein (*CP*) gene *AV1*. The amplified region corresponds to 576 bp, including primers (Ala-Poikela et al., 2005). To either 2 µl of FTA-extract, or to the preincubated PCR-tube, 23 µl PCR master mix was added. The PCR master mix contained 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.1 u of *Taq* DNA polymerase and 0.2 mM of each primer. PCR conditions were as follows: the samples were heated at 94 °C for 2 min, then subjected to 34 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min and extension at 72 °C for 2 min. The last cycle was followed by elongation at 72 °C for 10 minutes. The PCR products were visualized by electrophoresis (100 V) for 45 minutes on a 1.0% agarose gel stained with ethidium bromide in 1x TAE buffert.

2.4 Rolling circle amplification

In order to analyze if the infected tomato samples had mixed begomovirus infections, rolling circle amplification (RCA) was carried out on all samples that had positive PCR results from the third collection occasion (27th of May). DNA from the tomato tissue was extracted using the Extract-n-AmpTM Plant PCR kit (Sigma) as described previously in section 2.3. The RCA was carried out using the "Illustra TempliPhi 100 Amplification Kit" (GE Healthcare) according to the manufacturer's instructions. In brief, 5 µl sample buffer and 1 µl DNA-extract was heated to 95 °C for three minutes. When cooled to room temperature, 5 µl reaction buffer and 0.2 µl enzyme mix were added, and subsequently the reaction was incubated at 30 °C for 20 hours. As negative control, RCA was run without extracted DNA.

2.5 Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) was carried out in order to detect the number of viral genomes in the samples. Six samples from the 27th of May were successfully amplified by RCA: 4, 8, 12, 15, 18 and 20. Each sample was subsequently digested by the restriction enzymes *Sal*I, *Eco*RI and *Hpa*II (Fermentas), essentially according to the manufacturer´s instructions. One µl RCA product was digested by 10 u *Eco*RI, 10 u *Hpa*II and 1 FDU *Sal*I respectively. Digestion was carried out in a 37 °C heating block for two hours and subsequently inactivated in a 65 °C heating block for 10 minutes (*Sal*I) or 20 minutes (*Eco*RI, *Hpa*II). The RFLP products were analysed on a 1.0 % agarose gel stained with ethidium bromide at 100 V for one hour, using 1x TAE buffer.

3. Results

3.1 Detection of begomovirus infection

All samples from the 20 populations from the first sampling occasion tested negative in the PCR, thus no begomovirus infection at the age of three weeks was detected. From the second sampling occasion, PCR was run on samples from populations that showed symptoms of begomovirus infection: 6, 8, 10, 14 and 18. Two samples from population 11.1 were analyzed, one with clear symptoms and the other one with very mild symptoms. Samples from population 1 and 11.2 did not have symptoms and were randomly selected for PCR analysis. All samples were positive except one sample from population 11.1 (with clear symptoms) and the sample from population 1. The sample from population 11.2 was positive, however, the band was very weak. The samples from population 6, 8, 10, 14 and 18 were clearly positive. The PCR results from the third sampling occasion revealed clear bands at 576 bp in samples from all 20 populations, except for the sample from population 11.2 that had a very weak band and the sample from population 11.1 that was negative (**Fig. 3**). Thus, after two months all wild tomato populations had plants that were infected with begomovirus, except 11.1 where no begomovirus infection was detected.

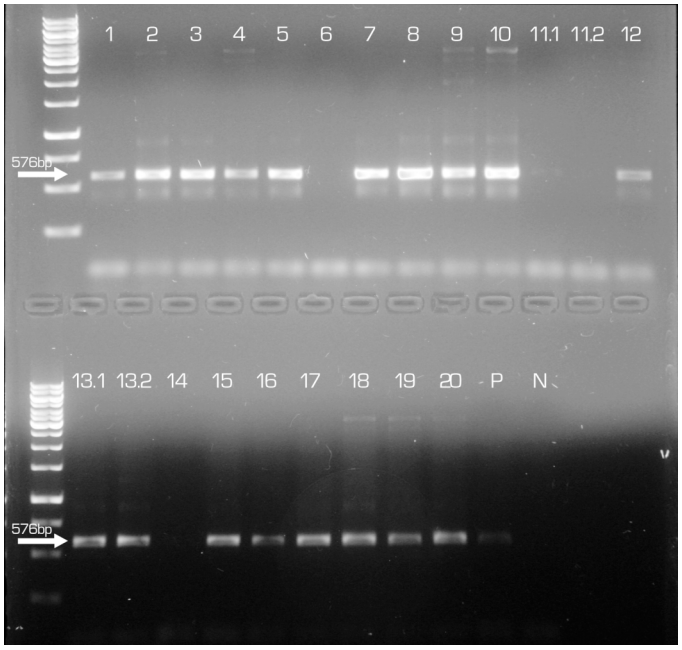

Fig. 3A. Polymerase chain reaction (PCR) detection of begomovirus using the primers AV494 and AC1048. The PCR products (576 bp) are from the third sampling occasion, when the tomato plants were two months old. The sample numbers in the figure correspond to the tomato population with the same number. The arrow indicates the PCR products at 576 bp. The size marker is 1 kb GeneRuler DNA Ladder (Fermentas).

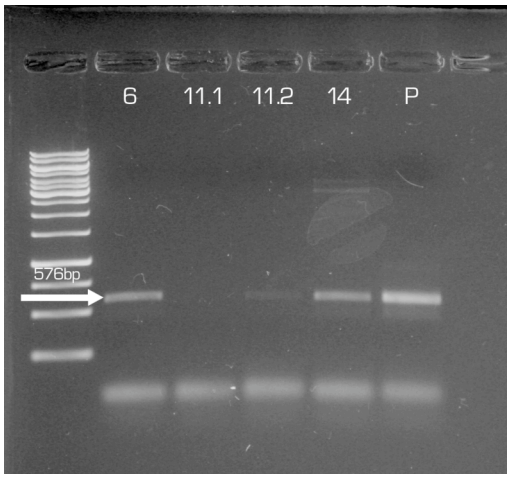

Fig. 3B. Rerun of negative samples from previous PCR (picture to the left).

3.2 Diversity of begomoviruses infecting wild tomato

All samples that were positive after PCR were amplified by RCA, in order to amplify the whole genome of the infecting begomoviruses. Six of the samples were successfully amplified by RCA, namely samples 4, 8, 12, 15, 18 and 20. Samples 4, 12 and 15 had very weak RCA products. Sample 20 had a relatively clear band, whereas sample 8 revealed a distinct RCA product (**Fig. 4**). The RCA product from sample 18 is not visible in Fig. 4, but was detected on another gel. The negative control was contaminated and indicated the presence of an RCA-product.

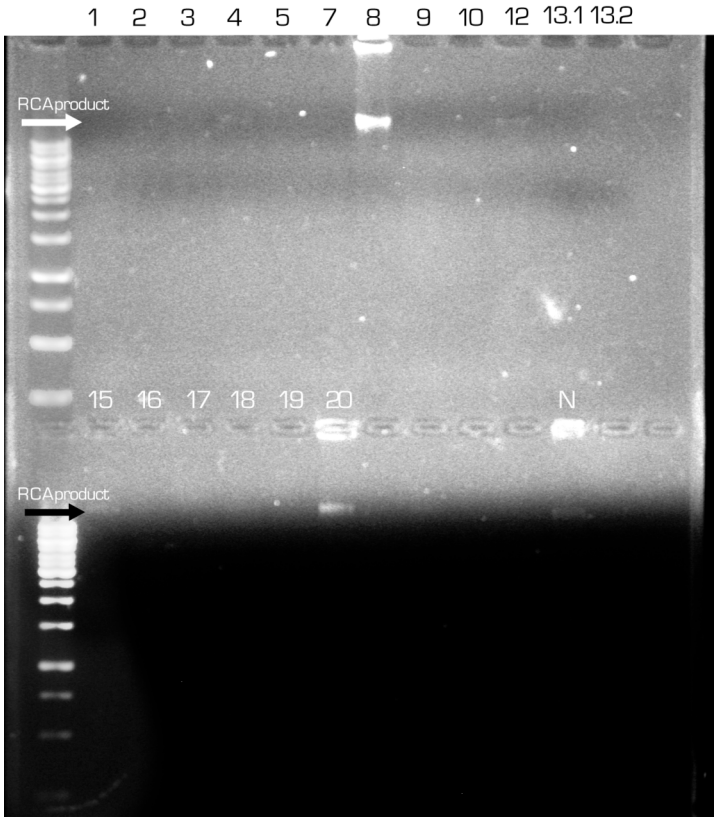

Fig 4. Rolling circle amplification (RCA) products. The sample numbers in the figure correspond to the tomato population with the same number. The marker is 1 kb GeneRuler DNA Ladder (Fermentas).

The RCA products of samples from population 4, 8, 12, 15, 18 and 20 were subsequently digested by the restriction enzymes *Sal*I, *Eco*RI and *Hpa*II, in order to detect mixed infections in the samples. The samples that had strong RCA-products had also distinct bands after the restriction analysis. Some bands are too weak to see in the figures, but were visible directly in the gel.

The restriction enzyme *Sal*I generated a full-length begomovirus fragment of approximately 2.7 kb in all samples (**Fig. 5**). For *Sal*I digestion of sample 18 see **Fig. 6**. A high molecular weight DNA band was still present in samples 8, 15, 18 and 20, although the fragment was very weak in all samples except sample 8 (**Fig. 5**). The result of the *Sal*I digestion indicated at least two viral DNA components in samples 8, 15, 18 and 20. One virus component was detected in samples 4 and 12.

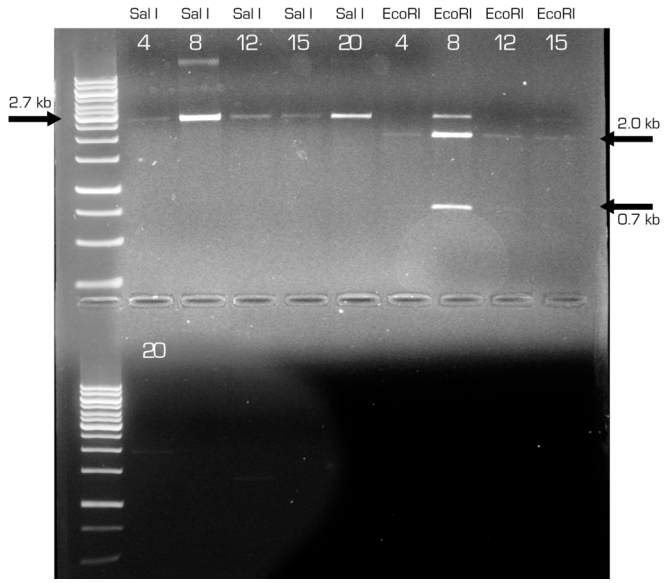

Fig 5A. Restriction digest analysis of RCA products of wild tomato samples. The sample numbers in the figure correspond to the tomato population with the same number. The marker is 1 kb GeneRuler DNA Ladder (Fermentas).

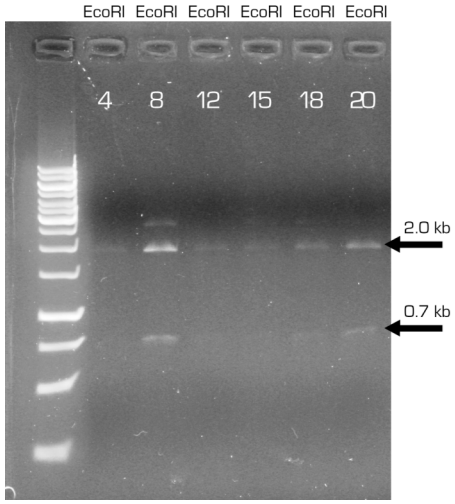

Fig 5B. Restriction digest analysis of RCA products of wild tomato samples. The sample numbers in the figure correspond to the tomato population with the same number. The marker is 1 kb GeneRuler DNA Ladder (Fermentas).

The restriction enzyme *Eco*RI generated fragments of 2.0 kb and 0.7 kb, which equals the size of a begomovirus DNA component, in all samples. In addition, full-length begomovirus fragments of approximately 2.7 kb were observed in sample 8, 15 and 18 (**Fig. 5A, 5B**). The result indicated the presence of two begomovirus components in sample 8, 15 and 18, whereas sample 4, 12 and 20 only appeared to have one component. Furthermore, the result of the *Eco*RI restriction cleavage supports the conclusion that the uncleaved RCA product after *Sal*I cleavage corresponds to one begomovirus component.

The restriction enzyme *Hpa*II generated fragments at 1.5 kb in samples 4, 8, 12, 15 and 20. In addition to the band at 1.5 kb, sample 8 had several bands of low molecular weight that approximately equal the size of a virus DNA component, 2.7 kb. In sample 20 an additional fragment at approximately 0.6 kb was detected. In sample 18, fragments of approximately 1.6 kb and 0.6 kb were visualized (**Fig. 6**). *Hpa*II generated very weak digestion patterns in sample 4, 12 and 15.

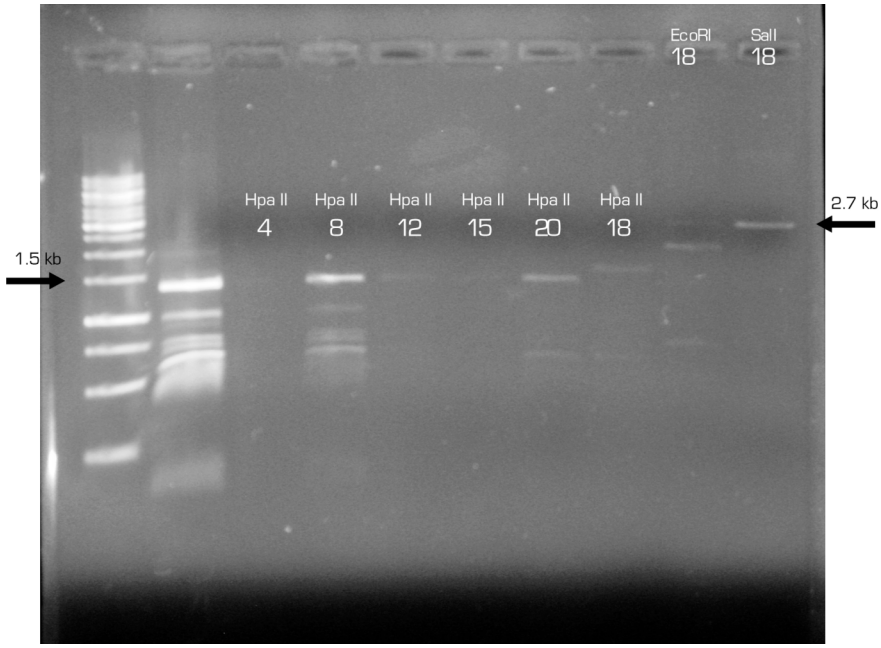

Fig 6. Restriction digest analysis of RCA products of wild tomato samples. The sample numbers in the figure correspond to the tomato population with the same number. The unmarked sample to the left is from another experiment. The marker is Fermentas 1 kb GeneRuler DNA Ladder.

The overall result of the restriction analysis indicated that mixed infections were not present in the investigated samples. At most, two virus components or one component and RCA product were detected. The outcome of the restriction analysis with each of the three enzymes is summarized in **Table 2**. The different digestions did not give consistent results. However, it is likely that samples 4 and 12 contained one begomovirus component, whereas samples 8, 15, 18 and possibly sample 20 contained two begomovirus components. Thus, all investigated samples were probably infected by one begomovirus.

Table 2. Summary of the outcome of restriction enzyme analysis of RCA products.

Sample	***Sal*I (kb)**[a]	***Eco*RI (kb)**	***Hpa*II (kb)**
4	2.7 (1)	2.7 (1)	1.5 (>1)
8	2.7 + RCA[b] (2)[c]	5.4 (2)	3.2 (<1)
12	2.7(1)	2.7 (1)	1.5 (>1)
15	2.7 + RCA (2)	5.4 (2)	1.5 (>1)
18	2.7 + RCA (2)	5.4 (2)	2.2 (>1)
20	2.7 + RCA (2)	2.7 (1)	2.1 (>1)

[a] The total size of the digested product is presented in kb.
[b] RCA means uncleaved RCA product in the sample.
[c] In parenthesis the putative number of begomovirus components is presented.

3. Discussion and Conclusions

The objective of this bachelor project was to investigate the begomovirus susceptibility in wild Nicaraguan tomato. Furthermore, as an additional aim the presence of mixed begomovirus infections in the wild plants was investigated.

The results of this project indicated that it is common that wild tomato plants are susceptible to begomovirus infection. During the time of this project it was possible to identify typical symptoms of begomovirus infection on tomato plants from each population. It seemed unlikely that any of the populations carried resistance genes based on their phenotypic appearance. On the contrary it was obvious that differences existed in severity of symptoms. Although all plants had been treated in the same manner the infection symptoms varied, which could indicate differences in plant defense between the populations. Some populations handled the infection better than others, which can imply genetic tolerance or resistance. The last PCR analysis, investigating the two months old tomato plants, showed that all samples were infected with begomovirus (except the sample from 11.1). Thus, it is evident that the plants were susceptible. The PCR also indicated possible differences in virus concentration between the samples. However, in order to determine differences in virus concentration, additional tests including more plant samples must be carried out and analyzed.

As for the population 11 (11.1 and 11.2) the situation was deviating. PCR analysis showed that plants from 11.2 were infected with begomovirus at the second and third sampling occasion. However, weak PCR products indicted very low virus concentrations compared to samples from the other populations. It is possible that 11.2 plants suppress the replication of the virus in some way. The tested plant from 11.1 was not infected with begomovirus at the third sampling occasion, and the plants in the field did not show begomovirus symptoms. However, at the second sampling occasion it was possible to find plants from 11.1 that had symptoms and the PCR analysis showed that one of the two tested plants was begomovirus susceptible. Nevertheless, it seems that 11.1 plants were not as easily infected as plants from the other populations. Plants from 11.1 showed very mild symptoms, if any, after an infection, which indicates a genetic advantage. Thus, the results of the tested plants from population 11 indicate signs of resistance or tolerance to begomovirus, or possibly resistance to the vector *B. tabaci*. In order to determine if population 11 could be used for breeding purposes, it is necessary to investigate if the plants have any of the genes previously found to be associated with begomovirus tolerance/resistance, or a previously unknown tolerance/resistance gene. This is in fact a necessary step to confirm that the population has any genetic advantages. When plants from many different populations are grown in the same area, it is possible that some of the susceptible plants escape infection even though the pressure from the vector *B. tabaci* is high. Screening for resistance properties in this manner could therefore be very misleading, a population that is not infected could falsely be taken as resistant. An alternative is to conduct artificial inoculation, when each plant is inoculated individually (Lapidot & Friedmann, 2002).

Some research states that a tolerant cultivar would be more suitable than a resistant cultivar in begomovirus management. In a resistant cultivar the virus replication is suppressed, which means higher selection pressure on the virus. Thus, new mutated strains of viruses may evolve, which means even higher diversity of begomoviruses (Seal et al., 2006). However, according to Rojas (2004) a resistant cultivar would be the best way to manage the begomovirus problem in Nicaragua. Regardless of the mechanism behind the mild disease symptoms it could be interesting to continue the investigation of population 11. Furthermore,

the coming research should definitely focus on continued screening of wild tomato plants in Nicaragua. Also, in a screening for resistance it is important to determine which begomoviruses the plants are infected by. By sequencing the viral genome it is possible to define which virus the specific plant has resistance or tolerance to. Even if the tomato genotype is resistant to one begomovirus it is not certain that it is resistant to other begomoviruses.

The second aim of this project was to test for mixed infections in the wild tomato plants. The results of this project indicated that no mixed infections of begomoviruses were present in the investigated tomato samples. It was not possible to detect more than one or two virus components (DNA-A and DNA-B) in the samples.

First of all, it is important to state that the RCA products were relatively weak, i.e. the concentration of circular DNA in the RCA product was low. This indicates either low virus concentration in the extracted tomato samples or an inefficient RCA. When comparing the same samples after PCR and RCA it is not possible to see any correlation; the same samples that gave weak PCR products were successfully amplified by RCA and tomato samples that gave strong PCR products were not successfully amplified by RCA. Thus, the RCA method appears to be somewhat random and more difficult to implement than PCR. However, it is possible that the virus concentration in the tomato samples was too low for an efficient RCA, which can be a reason why few samples gave an RCA product. Since the amplification from many of the samples were unsuccessful, RCA products with a presumably too low concentration were chosen for restriction analysis. Having weak RCA products, as in this project, more RCA product should have been used to ensure a more reliable restriction analysis. Moreover, filter tips should have been used in order to establish a more sterile environment and avoid problems such as the contaminated negative control. Also, it is important to mention that all DNA was assumed to be of begomovirus origin. The presence of e.g. RNA-viruses was not investigated in this project.

The low virus concentration in the RCA products can be an explanation to why some of the cleaved products (samples 4 and 12) only seemed to contain one begomovirus component. As mentioned before it is possible that the two components are present in somewhat different concentration in the infected tissue. It is possible that only the component with the higher concentration was amplified in a sufficient amount. Thus, the component identified in these samples after restriction analysis was probably DNA-A. Another possibility to the absence of a second component in some of the samples can be that the begomovirus TYLCV has come to Nicaragua. TYLCV does not have a B-component. This virus has previously been detected in countries like USA, Mexico and Cuba, but not in Nicaragua (Rojas, 2004). To determine if TYLCV is present in the infected tissue and has entered Central America, the viral genome must be sequenced.

The *Hpa*II digest patterns contained fewer base pairs than the digest patterns from the other enzymes; many of the *Hpa*II samples did not even contain a whole DNA component. It is very likely that many of the *Hpa*II fragments had similar sizes, which were not adequately separated on the agarose gel. In order to separate the fragments better one could have run the agarose gel at lower voltage and for longer time. Since *Hpa*II cleaves more frequently than *Eco*RI and *Sa*lI, it is easier to detect differences in digestion patterns between the samples, thus if they had different or identical components. When comparing *Hpa*II digestion patterns of the samples it was obvious that some fragments differed in size. Sample 18 had another digestion pattern than the other samples. This means that the digested components can be

derived from two different, but closely related, species of begomovirus. On the other hand, the *Eco*RI (and *Sal*I) digestion did not indicate the presence of different begomovirus species. The *Eco*RI digestion patterns gave rise to fragments of the same size. Moreover, it is not likely that different begomoviruses would have the same restriction site for *Eco*RI. However, it is possible that the *B. tabaci* population in Sebaco carries more than one species of begomovirus, which of course means a risk for future mixed infections. Previous research has found that mixed infection is common and a problem in Nicaragua (Ala-Poikela et al., 2005).

When screening for resistance it is important to gain knowledge about the pathogen, e.g., the number of begomovirus species present and the presence of mixed infections. Furthermore, since mixed infection is the most important process in begomovirus evolution it is a crucial process to observe, in order to carry out a successful management of the begomovirus problem in Nicaragua.

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