



ORIGINAL ARTICLE

Development of interspecific *Solanum lycopersicum* and screening for Tospovirus resistance



Sayed Sartaj Sohrab ^{a,*}, P.S. Bhattacharya ^b, D. Rana ^b, Mohammad A. Kamal ^a, M.K. Pande ^b

^a King Fahd Medical Research Center, King Abdulaziz University, Post Box No. 80216, Jeddah 21589, Saudi Arabia

^b Division of Biotechnology, JK Agri-Genetics Ltd., Hyderabad, A.P., India

Received 29 September 2014; revised 25 October 2014; accepted 7 November 2014

Available online 20 November 2014

KEYWORDS

Interspecific *S. lycopersicum*;
Embryo rescue;
Tospovirus resistance

Abstract Tospovirus has emerged as a serious viral pathogen for several crops including tomato. The tomato production is being severely affected worldwide by Tospovirus. Some reports have been published about the association of plant virus and development of human disease either by direct or indirect consumption. Resistance to this virus has been identified as good source in wild tomato species (*Lycopersicon peruvianum*). But the introgression of resistance genes into cultivated tomato lines and the development of interspecific hybrid are hampered due to incompatibility, fertilization barriers and embryo abortion. But this barrier has been broken by applying the embryo rescue methods. This study describes the development of interspecific hybrid tomato plants by highly efficient embryo rescue method and screening for Tospovirus resistance. The interspecific hybrid tomato plants were developed by making a cross between wild tomato species (*L. peruvianum*) and cultivated tomato (*Solanum lycopersicum*). The immature embryos were cultured in standardized medium and interspecific hybrids were developed from embryogenic callus. The immature embryos excised from 7 to 35 days old fruits were used for embryo rescue and 31 days old embryos showed very good germination capabilities and produced the highest number of plants. Developed plants were hardened enough and shifted to green house. The hybrid nature of interspecific plants

Abbreviations: GBNV/PBNV, Groundnut bud necrosis virus/Peanut bud necrosis virus; WBNV, Watermelon bud necrosis virus; TMV, Tobacco mosaic virus; MS, Murashige and Skoog's; ELISA, Enzyme linked Immunosorbant Assay

* Corresponding author at: Special Infectious Agents Unit, King Fahd Medical Research Center, King Abdulaziz University, Post Box No. 80216, Jeddah 21589, Saudi Arabia. Tel.: +966 554627872, +966 64001000x73530; fax: +966 6952521.

E-mail addresses: ssohrab@kau.edu.sa, sohrab_sartaj2@rediffmail.com (S.S. Sohrab).

Peer review under responsibility of King Saud University.



Production and hosting by Elsevier

was further confirmed by comparing the morphological characters from their parents. The F1, F2 and F3 plants were found to have varying characters especially for leaf type, color of stem, fruits, size, shapes and they were further screened for virus resistance both in lab and open field followed by Enzyme linked Immunosorbant Assay confirmation. Finally, a total of 11 resistant plants were selected bearing red color fruits with desired shape and size.

© 2014 The Authors. Production and hosting by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/3.0/>).

1. Introduction

Tomato (*Solanum lycopersicum*) is an important vegetable crop grown globally with an annual production close to 130 million tons in 2009. India is the third in tomato production (11.98 million tons in 0.69 million ha) worldwide (FAOSTAT, 2011). Currently, more than twenty Tospoviruses have been reported globally (Pappu et al., 2009; Mandal et al., 2012). The Tospoviruses (family *Bunyaviridae*) are enveloped isometric RNA viruses with three genomes designated as small (S), medium (M), and large (L) segments of ssRNA. Thrips (*Frankliniella fusca* Hinds, *F. occidentalis* Pergande) are the main vector for Tospovirus transmission (Mandal et al., 2012).

In India, Groundnut bud necrosis virus (GBNV) was reported in 1968 (Reddy et al., 1992; Mandal et al., 2012). Subsequently, new viruses were reported like; Peanut yellow spot virus (Satyanaryana et al., 1998) and Watermelon bud necrosis virus (WBNV) were reported in 1990 on groundnut and watermelon respectively (Jain et al., 1998) while, Iris yellow spot virus (Ravi et al., 2006) and Capsicum chlorosis virus reported on onion and capsicum during 2002–2006 (Krishnareddy et al., 2008; Mandal et al., 2012). Globally, twenty Tospoviruses have been reported on various crops like tomato, groundnut, watermelon, cucurbits, pepper, cowpea, mungbean, potato, chili and soybean and currently, five Tospovirus have been reported from India: GBNV/PBNV, WBNV, Capsicum chlorosis virus, Iris yellow spot virus and Peanut yellow spot virus on various crops like capsicum, groundnut, onion and watermelon (Kunkaliker et al., 2007, 2011; Jain and Mandal, 2009; Mandal et al., 2012). Development of Tospovirus resistant crops is an urgent need. The resistance in tomato can be achieved by introgression of the resistant genes from wild tomato (*Lycopersicon peruvianum*) to cultivated line. Breeding and host plant resistance for Tospovirus on tomato and peanut have been reviewed and published in many report (Canady et al., 2001; Mehdi and Sudhakar, 2008; David and Shimat, 2011; Sundaraj et al., 2014). Various resistant genes were identified and designated as Swa1, Swb1, Sw2, Sw3, and Sw4, Sw-5, Sw-6, Sw7 from wild source of tomato (*L. peruvianum*) against Tospovirus (Stevens et al., 1992, 2007; Ainong et al., 2011).

The interspecific F1 hybrids can be developed by interspecific crossing, but due to incompatibility barriers and early embryo abortion, it is not possible to obtain interspecific F1 hybrids by conventional methods. These barriers can be overcome by embryo rescue (Barbono and Topoleski, 1984; Pico et al., 2002). Several reports have been published about the successful development of interspecific hybrids by embryo rescue method and screening for Tospovirus resistance (Angora et al., 1981; Thomas and Pratt, 1981; Imanishi et al., 1985; Poysa, 1990; Chen and Adachi, 1992, 1996; Segeren et al., 1993a,b; Bhattarai et al., 2009; Encina et al., 2012; Kharkongar et al., 2013).

Globally, vector transmitted viruses are serious threat to plant, animal and human health which prompted the researchers to develop an efficient and highly effective control strategies for these viruses (Montero-Astua et al., 2014). Currently, some plant viruses belonging to families *Bunyaviridae*, *Rhabdoviridae* and *Reoviridae* are reported to infect and develop clinical disease symptoms in humans and it is expected that emergence or re-emergence of arboviruses had happened due to changes in climatic conditions especially in family *Bunyaviridae* (Le May and Bouloy, 2012). Some Plant viruses, such as Tospovirus, Rhabdovirus, Reovirus, Begomovirus and Nanovirus are suspected to have a minor host relationship with plants and animals (Mir et al., 2006; Hart et al., 2009; Mandal et al., 2012). Some reports have been published about the association of Plant viruses like Tobacco mosaic virus (TMV), Pepper mild mottle virus and Cowpea mosaic virus in human diseases like fever and abdominal pain causing health problems (Olszewska and Steward, 2003; Rae et al., 2005; Shriver et al., 2009; Colson et al., 2010; Mandal and Jain, 2010; Liu et al., 2013).

The above information suggests that plant virus can infect human and play very important role in disease progression. Thus, there is an urgent need to develop virus resistant crops especially for the fruits and vegetable crops as human beings are consuming raw fruits in various ways. Thus, to meet the demand for successful development of interspecific F1 tomato plants for Tospovirus resistance and to overcome the fertilization barriers, this work was carried out by crossing the cultivated tomato line *S. lycopersicum* (492-BC), with *L. peruvianum* (921L00671). Embryo rescue was conducted to culture immature embryo isolated from crossing fruits of *S. lycopersicum* (492-BC) and *L. peruvianum* peruvianum (921L00671) and the F1 hybrid tomato plants were developed. The selected hybrid plants were confirmed by differentiating morphological character with their parents and the Tospovirus resistance was confirmed by virus inoculation and Enzyme linked Immunosorbant Assay (ELISA).

2. Materials and methods

2.1. Seed germination and raising of tomato seedlings

The experimental material used in the study comprised of species viz., cultivated tomato *S. lycopersicum* (492-BC) and wild *L. peruvianum* (921-L00671). Selected seeds of both tomato plants were germinated at room temperature in germinating trays having a mixture of manure, soil and sand (1:1:1 ratio) and mixture was treated with fungicides like Captan (0.1%) and Carbendazim (0.05%) to prevent seedling diseases. Four weeks old seedlings were transplanted into big pots and kept under screen house for further use.

2.2. Selection and interspecific crossing

The cultivated tomato *S. lycopersicum* (492-BC) as female parent and wild *L. peruvianum* (921-L00671) was selected as male parent in this work. The crossing was made in the month of March–April. Emasculation of female parents was done by hand just prior to one day before anthesis at approximately 3–4 PM and their pistils were covered by using butter paper bags to avoid contamination from foreign pollen. The female stigma was pollinated next day, in between 7 and 9 AM by using the freshly collected pollens from the male parent. The stigma was again covered and allowed for fertilization and fruits setting.

2.3. Collection of fertilized tomato fruits and isolation of immature embryos

After crossing, hybrid fruits at different developmental stages (7, 14, 21, 28, 29, 31, 33, 35 days after pollination) were harvested and washed with distilled water for 5 min and then sterilized with Sodium hypochlorite (2.5%) solution for 30 min and ethanol (100%) for 5 min followed by 6 times washing with sterilized distilled water. A total of 15–20 immature embryos were cultured/plate. Total, 4160 immature embryos were isolated aseptically from 557 fruits and cultured on Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962) for embryo rescue and their regeneration rate was observed periodically.

2.4. In-vitro culture: Embryo rescue and development of interspecific F1 hybrid tomato

The immature embryos were excised from sterilized tomato fruits and cultured for 25 days on MS medium with 2.0 mg/L Benzyl aminopurine 1 gm/Yeast extract, Sucrose (3%) and medium was solidified with Gelrite (0.3%). The cultured embryos were kept in the culture room for germination. The temperature was maintained at $25 \pm 2^\circ\text{C}$, with humidity of 65% and under a relative humidity of 60%. The regular observations of cultures were made for recording data of proper germination. The best growth response was observed in the embryos isolated from immature fruits of 28–33 days of pollination. The cultured embryos started to shoot induction only after 20 days on the shoot induction medium. The shoot inducing embryos were further sub-cultured on shoot multiplication MS medium with 2.0 mg/L Benzyl aminopurine and 0.1 mg/L Indole-3-butyric acid (IBA) for 20 days. The regenerated shoots (2–3 cm long) were isolated and sub-cultured on MS medium for shoot elongation without any growth hormones. The elongated shoots were further sub-cultured on $\frac{1}{2}$ MS medium free from growth hormones for root formation till 20 days. Finally, shoots with profuse roots were hardened in $\frac{1}{2}$ MS liquid medium for 10 days. The developed plantlets were shifted in small cups with peat moss mixture, vermiculite and lignite (1:1:1 ratio) in the culture room until transplanted and covered with Polythene bags with small openings for 15 days. The acclimatized plantlets at 5–6 leaf stage were transferred to big pots containing soil, farm yard manure with sand and cultured under green house till flowering and fruit formation.

2.5. Morphological evaluation of F1 hybrids

The hybrid nature of F1, F2 and F3 plants were evaluated by visual morphological observation of parents and their hybrid plants on the basis of leaf shapes, leaf size, stem diameter, flowers, stigma exertion, stem color, fruit's shape, size, color, presence and absence of leaf and stem trichomes and internodes length.

2.6. Formation of F1 fruits, seed setting and harvesting of F2 seeds

All F1 plants were grown in big pots and their cuttings were made, grown in natural field conditions to produce fruits and seed setting. All plants produced a large number of flowers which were sib-mated to produce large numbers of F2 generations fruits and seeds. This process was adopted to increase the chances of getting more fruits and seed setting, recovering multiple gene combinations that provide better agronomic characters with broader spectrum disease resistance. A total of 533 F1 tomato fruits were harvested from various plants (28, 29, 31 and 33 days old).

2.7. Germination of F2 seeds and harvesting of F3 seeds

Fully matured and ripen fruits from F1 plants were harvested and excised to obtain F2 seeds. The well dried F2 seeds were directly sown in autoclaved soil and allowed to germinate properly. Large number of F3 seeds were recovered from ripens fruits and further used for direct sowing and screening in the field and desired tomato plants were selected bearing red fruits with bigger size and good shape.

2.8. Culture and maintenance of pure virus

Pure culture of Tospovirus (PBNV) was maintained on Cowpea (Var. Pusa Komal) which is a good indicator and multiplication host for Tospovirus. Tospovirus infected tomato fruits were collected from infected plants in the field (Fig. 1). The virus infections were identified primarily on the basis of rings symptoms on fruits, leaf and stem necrosis of infected plants and further confirmed by ELISA by using PBNV specific antibodies. The virus inoculum was prepared by using freshly harvested Tospovirus infected tomato from the field. The infected tomato fruit pericarp tissue (5 gm) was grounded in 5 ml 0.1 M Sodium Phosphate buffer (w/v), pH 7.2 supplemented with sodium sulfite (0.1%). The virus inoculum was filtered through cotton pad and a pinch of Celite was added.

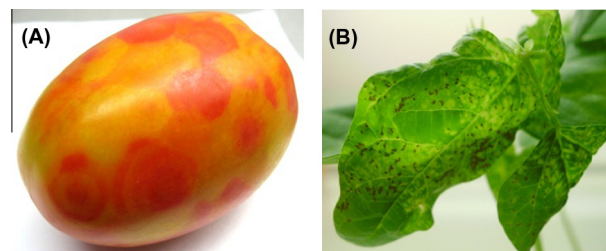


Figure 1 Inoculation and symptoms of PBNV on Cowpea. (a) Naturally infected tomato. (b) Inoculated Cowpea.

Finally the virus inoculum was applied on the first true leaves about 6–7 days old healthy cowpea seedlings pre-dusted with carborundum powder. The inoculated plants were washed with tap water before drying the leaves and kept under shade in green house up to 7–15 days for symptom expression. After symptoms development, the virus was further inoculated on freshly raised cowpea seedlings and pure virus culture was maintained under insect proof glass house and used further for virus inoculation on tomato plants.

2.9. Screening and selection of tomato plants resistance to Tospovirus

To standardize the sap transmission of Tospovirus from naturally infected tomato, an attempt was made to transmit the virus on tomato and cowpea seedlings from various types of inoculum sources like infected tomato leaves, tomato fruits pericarp and infected cowpea leaves. Infected tomato fruit pericarp was observed as the best source of virus inoculum. The virus infection was observed on inoculated Cowpea seedlings (100%) and tomato (60–70%) as compared to inoculum used from the leaves of tomato and cowpea. On the basis of results obtained, tomato fruits pericarp as an inoculum source was selected for further study. For screening of F1 hybrids and F2, F3 segregating tomato resistant to Tospovirus, virus inoculation was done with F1 cuttings by using freshly prepared inoculum from naturally infected tomato fruits pericarp. Mechanical inoculation was done primarily by sap and 3 days post inoculation, second step inoculation was done by viruliferous Thrips. Thrips were given acquisition feeding on infected cowpea for 3–5 days and released on inoculated tomato plants for virus inoculation in a closed chamber. After 15 days of first inoculation, third step inoculation was done by sap and transplanted in natural open field for symptoms development.

Before transplanting of inoculated hybrids of F1, F2 and F3 tomato plants, natural field was prepared by sowing the cowpea and susceptible tomato plants. The sap inoculated Cowpea plants showing symptoms were transplanted near to Cowpea and tomato plants in the field and thrips were released to spread the virus on healthy tomato and Cowpea plants. After full symptom development on Cowpea and tomato plants, the inoculated hybrid tomato plants were transplanted to open field for natural virus infection. The susceptible tomato and Cowpea plants were also sown in between the rows of inoculated hybrids plants. The evaluation was done on the basis of symptom expression and resistance was confirmed by ELISA. The criteria for selection of desired tomato plants were as: phenotypic resistance (susceptible, tolerant, immune), phenotypic character of plant (cultivated/wild type), Tospovirus infection (yes/no), flower setting/dropping, fruit setting/dropping, fruit shape, size, color (after ripening), seed setting, ELISA (+/–) & (Triple confirmation by ELISA after 10 and 15 days), tolerance to other virus infection like cucumber mosaic virus and Tomato leaf curl virus. The resistant plants were further evaluated and selected on the basis of their fruit shape, size and color.

2.10. ELISA

The virus infection was confirmed by using the protocol for (Clark and Bar-Joseph, 1984) direct antigen coating- Enzyme

linked Immunosorbant Assay in 96 well polystyrene plate (Sigma, USA). The symptomatic tomato fruit tissues and leaves were ground at 1:1 dilution (w/v) in extraction buffer (sodium carbonate buffer, pH 9.6) with 2% Polyvinylpyrrolidone (MW 40,000), filtered through cotton pad. Supernatant (150 µl/well) was used to load ELISA plates and incubated for 1.5 h at 37 °C with gentle shaking after 10 min interval. Extracts from PBNV infected and healthy tomato were used as positive and negative control. PBNV Polyclonal antiserum was used at 1:5000 dilutions after cross absorption with healthy tissue. After washing of plates, anti-rabbit immunoglobulin ALP (Alkaline Phosphatase) conjugate (Sigma Chemical Co., St. Louis, USA) was used at 1:20,000 dilutions. The ELISA reactions were read at 405 nm 1 h after adding substrate *p*-nitro phenyl phosphate 0.5 mg/ml, Sigma Chemical Co., St. Louis, USA) by using a ELISA reader.

3. Results

3.1. Seed germination and rising of tomato seedlings

The seed germination rate of both tomato species *S. lycopersicum* (492-BC) and wild *S. peruvianum* (921L00671) were observed to be 100%. The germinated seedlings were grown under insect proof screen house. The four weeks old seedlings were transplanted into pots and open field.

3.2. Interspecific crossing and fruit set

The efficient introgression of important characters from wild to cultivated tomato have been hindered by both pre-zygotic and post-zygotic interspecific crossing barriers between *S. lycopersicum* and *S. peruvianum* (Barbono and Topoleski, 1984). In our study, interspecific cross between *S. lycopersicum* (492-BC) and *S. peruvianum* (921-L00671) was observed to be normal and good fruits setting happened but embryo abortion and seedless fruits were also observed. Normal pollen germination was recorded in selfing, interspecific crosses of cultivated species with wild species. Similar, normal pollen germination was reported earlier in the interspecific crosses of *S. lycopersicum* and wild tomato (*S. chilense*, *S. peruvianum* and *S. hirsutum*) (Pico et al., 2002). The reduced pollen fertility after prolonged periods of high temperature in the field was also observed (Dane et al., 1991; Abdul-Baki and Stommel, 1995).

3.3. Tomato fruits collection and isolation of immature embryos

Tomato flowering, fruit setting and seed formation is highly depend on various factors like; pollen viability, time of pollination, optimum moisture and humidity, successful fertilization, and day/night temperature. The best temperature for more number of fruit setting and seed formation was found in a range between 15 °C and 20 °C (Dane et al., 1991). In our study maximum fruit set was observed and total 850 tomato fruits were collected from crossed plants at different time intervals (7, 14, 21, 28, 29, 31, 33, 35 days after pollination) (Table 1). During isolation of immature embryos it was observed that most of the fruits were empty and only, 557 fruits were found to have good immature embryo in 28–33 days old fruits. None of the immature embryos were found

Table 1 Total no of interspecific F1 tomato plants developed.

Tomato fruits (days after pollination)	Total number of fruits harvested	Total number of plants developed	Number of cuttings made
28	135	8	40
29	142	12	60
31	171	24	120
33	109	5	25
Total	557	49	245

in 7–21 days old fruits. In some fruits we have also observed the brown wrinkle deformed seeds which indicate the post fertilization abnormalities happened during crossing and fertilization. The same kind of observations has been reported earlier (Kharkongar et al., 2013). Total, 4160 immature embryos were isolated aseptically from 557 tomato fruits harvested after 28–33 days after pollination.

3.4. In-vitro culture: Embryo rescue and development of tomato plant

In our study, it was observed that 31 days old immature embryos are the best stage for embryo rescue as it produced the highest number of interspecific F1 plants (Table 1). The barriers of successful fertilization and development of interspecific hybrids have been overcome by various alternative methods like embryo rescue and normal seed development with proper management of crossing and important environmental factors required for better fruit set and seed formation, pollination with gamma-ray irradiated pollen grain, use of polyploidy bridge crossing and the selection of self-compatible species (Poysa, 1990). The interspecific hybrids have also been developed by ovule culture (Imanishi et al., 1985) and ovule derived callus (Thomas and Pratt, 1981). The age and correct composition of the growth has a great influence on the success of rescuing the hybrids. Various researchers have published about variable level of germination at different media combinations (Chen and Adachi, 1992, 1996; Segeren et al., 1993a,b; Hossain et al., 2003; Bhattarai et al., 2009; Kharkongar et al., 2013). In our study, the MS medium with 2.0 mg/L Benzyl aminopurine, 1 gm/Yeast extract, Sucrose (3%) was observed as the best medium for embryo rescue. The germinated embryo formed callus and multiple shoots were emerging from the callus. The developed shoots had profusely formed roots and they were hardened and further utilized (Fig. 2).

3.5. Morphological characteristics of F1 hybrids

Morphological variations of interspecific hybrids have been already described by other researchers (Thomas and Pratt, 1981; Imanishi, 1988). A great deal of morphological variations was found and our observations strongly supported that the developed interspecific F1 hybrid plants also have different morphological variations which are more similar to their parents. Most of the leaves were of variable shapes, slightly bigger than their parents; presence of leaf hairs, purple stem color in some plants, and presence of trichomes on stem, broader stem diameter and intermodal length was more as compared to their

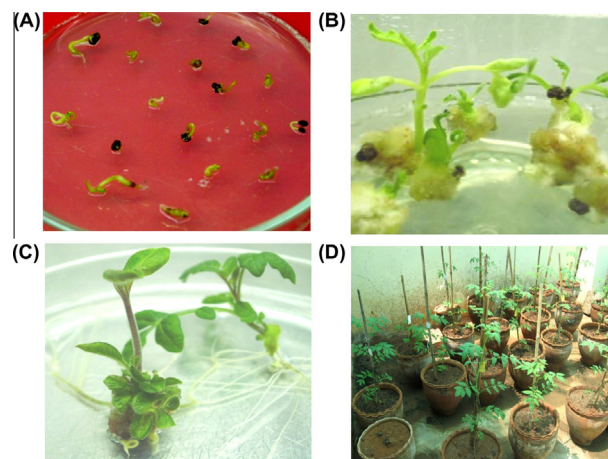


Figure 2 Various stages of interspecific F1 tomato plant development. (A) Germinating immature embryos. (B) Shoot induction. (C) Shoot elongation and rooting. (D) Regenerated plants in pots.

parents. Some plants found to be more toward cultivated and less wild type and their height and length were more than cultivated parent. In some plants, flower formation and fruits settings were very less. Some plants produced variable color fruits (orange light red and light yellow color). Some plants were observed as susceptible to Cucumber mosaic virus and Tomato leaf curl virus infection. All F1 hybrids produced intense yellow color flower with exerted stigma, a number of bigger fruits/plant and their shapes were slightly different from wild fruits (Fig. 3). Fruit color was green but turned into orange color once ripen but none of the fruits turned fully red as the female parent.

3.6. Formation of F1 fruits, seed setting and harvesting and germination of F2 seeds

All the F1 plants produced a large number of fruits with less number of mature seeds. The F2 seeds were collected from F1 hybrid tomato fruits (Fig. 3). A total of 533 F1 tomato fruits were harvested from various plants. The size of F1 tomato fruits was slightly bigger than the wild tomato and smaller than the cultivated tomato. The color of tomato fruits was yellow and slightly orange after ripening. The harvested F1 fruits were used for isolation of F2 seeds from fully matured and ripen tomato fruits. All the F2 seeds were germinated well and converted into full plants. A large number of bigger size fruits and more number of seed set were observed in F2 generation. In F3 generation, various plants developed red color fruits with desired shapes and size with more seed setting. The selected F4 plants were further evaluated and screened by virus inoculation and resistance.

3.7. Culture and maintenance of pure virus

Tospovirus infected tomato fruits were collected from infected plants in the field. The virus was easily transmitted through sap inoculation by using inoculum prepared from tomato fruit pericarp on cowpea and tomato plants. After 3–4 days of

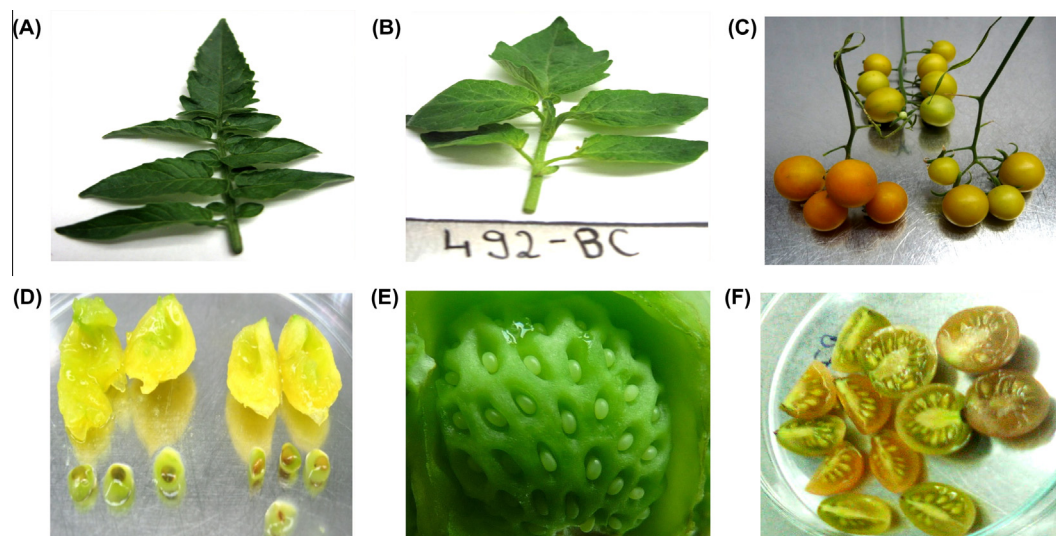


Figure 3 Morphological variations of interspecific plants. (A) Leaf (Wild). (B) Leaf (Cultivated). (C) F1 fruits. (D) F1 seeds. (E) F2 seeds. (F) F3 seeds.

Table 2 Total number of infected/inoculated plants and resistant plants found after ELISA.

Tomato fruits (days after pollination)	Total number of infected/inoculated plants						ELISA (No. of resist. plants)
	F1		F2		F3		
	1st Week	8th Week	1st Week	8th Week	1st Week	8th Week	
28 days old	1/40	21/40	2/21	10/21	1/11	9/11	2
29 days old	3/60	36/60	2/24	11/24	2/13	8/13	5
31 days old	9/120	81/120	3/39	26/39	4/13	10/13	3
33 days old	1/25	14/25	1/11	6/11	1/5	4/5	1
Susceptible plant (492-BC1)	21/344	340/344					
Wild plant (<i>L. Peruvianum</i>)	0/121	0/121					

inoculation, localized symptoms appeared on cowpea (Chlorotic local lesion) which converted into systemic symptoms (Necrotic lesion) within 5–6 days (Fig. 1).

3.8. Screening and selection of interspecific F1 tomato plants resistance to Tospovirus

Mechanical inoculation of Tospovirus on cultivated tomato was found to be susceptible under green house. Large number of F1 plant was found to be resistant to PBNV with variable degree. It was observed that, hundred percent plants produced symptoms after thrips inoculation. The F2 and F3 plants were selected on the basis of number of plants infected/inoculated, the degree of resistance and ELISA result. In F4 generation only 11 plants selected as resistant on the basis of visual observation, virus inoculation and ELISA confirmation (Table 2).

3.9. ELISA

The collected samples of tomato fruits and leaves efficiently reacted with polyclonal antiserum of PBNV in ELISA, which supported concept the infection of a Tospovirus is antigenically related to PBNV. The virus was detected both in fruits and leaves with PBNV antiserum.

4. Discussion and conclusion

This study describes the successful development of interspecific hybrid F1 tomato by embryo rescue and introgressed the Tospovirus resistant genes in cultivated tomato by crossing with wild species (*L. peruvianum*) and screened for Tospovirus resistance. The screening was done by sap and thrips inoculation and resistant plants were further confirmed by ELISA with PBNV antibody. The developed and selected F4 plants showed a higher degree of resistance against PBNV and produced red color fruits with good shape and bigger size. Globally, the production of tomato is greatly affected by Tomato spotted wilt virus. So the search for resistance against this disease has prompted the researchers to identify the resistance in various genetic resources, especially wild tomato species (Kalloo, 1986). The resistance to Tospovirus is mainly controlled by single and mostly dominant and recessive genes. Many reports have been published about the identification of resistant genes; Swa1, Swb1, Sw2, Sw3, and Sw4, Sw-5 and Sw-6 (Ainong et al., 2011) and their relationships to provide broad spectrum resistance against Tospovirus (Canady et al., 2001; Aramburu and Marti, 2003; Mehdi and Sudhakar, 2008; David and Shimat, 2011; Sundaraj et al., 2014).

The successful transfer of resistant genes from wild species to cultivated tomato by conventional methods are very

difficult and gets affected due to interspecific incompatibility and early embryo abortion, but this problem was overcome by embryo rescue (Pico et al., 2002; Bhattarai et al., 2009; Encina et al., 2012; Kharkongar et al., 2013). The development of interspecific hybrid plants by applying embryo rescue methods have been published in many reports (Angora et al., 1981; Thomas and Pratt, 1981; Imanishi et al., 1985; Poysa, 1990; Chen and Adachi, 1992; Segeren et al., 1993a,b; Bhattarai et al., 2009; Encina et al., 2012; Kharkongar et al., 2013).

In our study, we observed that germinated embryo formed callus and developed lots of multiple shoots on the standardized MS medium. No hormones were required for shoot elongation and which supports that this standardized protocol is more simple than other reports in which various kinds of hormones are required for shooting, shoot elongation and rooting (Cap et al., 1991; Segeren et al., 1993b; Chen and Adachi, 1996; Dan et al., 2006; Sun et al., 2006; Rao et al., 2005; Bhattarai et al., 2009; Encina et al., 2012; Kharkongar et al., 2013). The developed interspecific hybrids showed a greater degree of morphological variations especially leaf morphology, flower types (exserted stigma), fruit's shape, size, and colors, empty fruits due to embryo abortions, stem diameter, presence of trichomes on stem and leaves, number of seeds/fruits, number of fruits setting/plants. This morphological variation have also been observed and reported by other researchers which strongly supports our observations and findings (Chen and Adachi, 1992, 1996; Segeren et al., 1993a,b; Bhattarai et al., 2009; Encina et al., 2012; Kharkongar et al., 2013).

The screening of F1 and F2 hybrids for disease resistance has been published earlier (Segeren et al., 1993b). During our study, we inoculated the developed plants by sap as well as thrips inoculation in the lab as well as in open field conditions. Variable degree of resistance and symptoms were observed. Some plants developed necrosis symptoms on inoculated leaves while others produced on newly emerging leaves. But some plants were non symptomatic but they were found to be infected with the virus, confirmed by ELISA using upper and lower leaves even after 45–50 days of inoculation. We did highly selective screening of resistant plants based on various parameters as mentioned in materials and methods. During phenotypic screening, we found that some plants were showing symptoms as like Cucumber mosaic virus and Tomato leaf curl viruses and it is possible that some other viruses can also infect the plants. Some plants developed a large number of bigger fruits with red color. Finally few resistant plants were selected with desired characters as like cultivated tomato plants.

There are published reports about the Tospovirus infected cucurbits, tomato, pepper, lettuce and chili are used by human as salad. The reported viruses are capable to switch their hosts easily to higher animals, human and replicate human cell lines and their vectors (de Medeiros et al., 2005; Mandal and Jain, 2010). Recently, the presence of Pepper mild mottle virus has been reported from food products (57%), adults and children stool samples (7.2% and 0.7%) and some clinical symptoms like fever, abdominal pains were observed in those patients having Pepper mild mottle virus (Zhang et al., 2006; Colson et al., 2010). TMV is known to infect tobacco, highly contagious and stable in dead tobacco tissues sputum and thoracentesis fluids of cigarette smokers and smokeless tobacco (Mandal and Jain, 2010; Liu et al., 2013). Cigarette smoking causes various types of disease like cancers (Hymowitz,

2011), heart disease (Prochaska and Hilton, 2012), rheumatoid arthritis (Miller et al., 2012) and multiple sclerosis (Emre and Decker, 1992; Sopori, 2002; Arnson et al., 2010), neurodegenerative disorders and lower risk of developing Parkinson's disease (Checkoway et al., 2002; Ritz et al., 2007; Powers et al., 2008; Chen et al., 2010; Quik et al., 2012). Higher level of Anti-TMV antibodies (IgG, IgG1, IgG3, IgG4, IgA, and IgM) have been detected by ELISA in cigarette smokers than nonsmokers. Risk of Parkinson's disease development has been observed in cigarette and their products smoking patients. The mechanism of molecular relationship between human disease and plant viruses should be explored and explained in the near future with strong evidence (Liu et al., 2013). The production of antibodies against cowpea mosaic virus was reported without replication in mice which enters through the intestinal bloodstream (Olszewska and Steward, 2003; Rae et al., 2005; Shriver et al., 2009).

In conclusion, we have successfully introgressed the resistant genes into cultivated tomato and developed interspecific tomato plant by embryo rescue showing good resistance against PBNV and bearing big size, red color and desired shape tomato fruits.

Conflict of interest

The authors confirm that there is no conflict of interest for the information presented in this manuscript.

Acknowledgements

Authors extend their thanks to Mr. SK Gupta (CEO, JK Agri-Genetics Ltd. Hyderabad, India) for providing funds, necessary facilities, guidance, valuable suggestions and continuous encouragement.

References

- Abdul-Baki, A.A., Stommel, J.R., 1995. Pollen viability and fruit set of tomato genotypes under optimum- and high-temperature regimes. *HortScience* 30, 115–117.
- Ainong, S., Richard, V., Richard, G., Pengyin, C., Homer, C., Dilip, P., 2011. Identification of molecular markers for Sw-5 gene of tomato spotted wilt virus resistance. *Am. J. Biotechnol. Mol. Sci.* 1 (1), 8–16.
- Angora, G., Saccard, F., Cappadocia, M., Ramulu, K., 1981. Backcross progenies from *L. esculentum* × hybrid (*L. esculentum* × *L. peruvianum* Mill.). *ZPflzucht* 87, 153–157.
- Aramburu, J., Marti, M., 2003. The occurrence in north-east Spain of a variant of Tomato spotted wilt virus (TSWV) that breaks resistance in tomato (*Lycopersicon esculentum*) containing the Sw-5 gene. *Plant Pathol.* 52, 407.
- Arnson, Y., Shoenfeld, Y., Amital, H., 2010. Effects of tobacco smoke on immunity, inflammation and autoimmunity. *J. Autoimmun.* 34, J258–J265. <http://dx.doi.org/10.1016/j.jaut.2009.12.003>.
- Barbono, P.P., Topoleski, L.D., 1984. Post-fertilization hybrid seed failure in *L. esculentum* × *L. peruvianum* ovules. *J. Am. Soc. Hort. Sci.* 109, 95–100.
- Bhattarai, S.P., de la Pena, R.C., Midmore, D.J., Palchamy, K., 2009. In vitro culture of immature seed for rapid generation advancement in tomato. *Euphytica* 167, 23–30.
- Canady, M.A., Stevens, M.R., Barineau, M.S., Scott, J.W., 2001. Tomato Spotted Wilt Virus (TSWV) resistance in tomato derived from *Lycopersicon chilense* Dun. LA 1938. *Euphytica* 117 (1), 19–25.

- Cap, G.B., Roberts, P.A., Thomason, I.J., Murashige, T., 1991. Embryo culture of *L. esculentum* X *L. peruvianum* genotypes possessing heat stable resistance to *M. incognita*. *J. Am. Soc. Hort. Sci.* 116 (6), 1082–1088.
- Checkoway, H., Powers, K., Smith-Weller, T., Franklin, G.M., Longstreth Jr., W.T., 2002. Parkinson's disease risks associated with cigarette smoking, alcohol consumption, and caffeine intake. *Am. J. Epidemiol.* 155, 732–738.
- Chen, L., Adachi, T., 1992. Embryo abortion and efficient rescue in interspecific hybrids, *Lycopersicon esculentum* and the 'peruvianum-complex'. *Jpn. J. Breed* 42, 65–77.
- Chen, L., Adachi, T., 1996. Efficient hybridization between *Lycopersicon esculentum* and *L. peruvianum* via 'embryo rescue' and in vitro propagation. *Plant Breeding* 115, 251–256.
- Chen, H., Huang, X., Guo, X., Mailman, R.B., Park, Y., 2010. Smoking duration, intensity, and risk of Parkinson disease. *Neurology* 74, 878–884.
- Clark, M.F., Bar-Joseph, M., 1984. Enzyme linked Immunosorbant Assays in plant virology. In: Maramorosch, K., Koprowski, H. (Eds.), . In: *Methods in Virology*, vol. II. Academic Press, New York, pp. 51–58.
- Colson, P., Richet, H., Desnues, C., Balique, F., Moal, V., 2010. Pepper mild mottle virus, a plant virus associated with specific immune responses, Fever, abdominal pains, and pruritus in humans. *PLoS One* 5, e10041. <http://dx.doi.org/10.1371/journal.pone.0010041>.
- Dan, Y.H., Yan, T., Muniyikwa, J., Dong, Y., Zhang, Armstrong, C.L., 2006. MicroTom-a high throughput model transformation system for functional genomics. *Plant Cell Rep.* 25, 432–441.
- Dane, F., Hunter, A.G., Chambliss, O.L., 1991. Fruit set, pollen fertility, and combining ability of selected tomato genotypes under high temperature field conditions. *J. Am. Soc. Hort. Sci.* 116, 906–910.
- David, G.R., Shimat, V.J., 2011. Host Plant Resistance to Tomato spotted wilt virus (*Bunyaviridae: Tospovirus*) in Tomato. *Hort-Science* 46 (12), 1626–1633.
- De Medeiros, R.B., Figueiredo, J.de.O., Resende, R.De., Avila, A.C., 2005. Expression of a viral polymerase-bound host factor turns human cell lines permissive to a plant- and insect-infecting virus. *Proc. Natl. Acad. Sci. U.S.A.* 102, 1175–1180.
- Emre, M.de., Decker, C., 1992. Effects of cigarette smoking on motor functions in patients with multiple sclerosis. *Arch. Neurol.* 49, 1243–1247.
- Encina, C.L., Carmona-Martin, E., Fernandez-Munoz, R., 2012. Embryo rescue of *Solanum lycopersicum* X *S. peruvianum* tomato cross for TOCV resistance. *Acta Hort. (ISHS)* 935, 91–93.
- Hart, T.J., Kohl, A., Elliott, R.M., 2009. Role of the NSs protein in the zoonotic capacity of Orthobunyaviruses. *Zoonoses Public Health* 56 (6–7), 285–296.
- Hossain, M.A., Minami, M., Nemoto, K., 2003. Immature embryo culture and interspecific hybridization between *Capsicum annuum* L. and *C. frutescens* L. via embryo rescue. *Jpn. J. Tropical Agric.* 47, 9–16.
- Hymowitz, N., 2011. Smoking and cancer: a review of public health and clinical implications. *J. Natl. Med. Assoc.* 103, 695–700.
- Imanishi, S., 1988. Efficient ovule culture for the hybridization of *Lycopersicon esculentum* and *L. peruvianum*, *L. glandulosum*. *Jpn. J. Breed* 38, 1–9.
- Imanishi, S., Watanabe, Y., Hiura, I., 1985. A simple and efficient method for the interspecific hybridization between *Lycopersicon esculentum* and *L. peruvianum*. *J. Yamagata Agric. Forestry Soc.* 42, 13–15.
- Jain, R.K., Mandal, B., 2009. Present status of chronic and emerging Tospoviruses in India. IXth International symposium on Thysanoptera and Tospoviruses 31August-4 September. *J. Insect Sci.* 10, 166.
- Jain, R.K., Pappu, H.R., Pappu, S.S., Krishnareddy, M., Vani, A., 1998. Watermelon bud necrosis Tospovirus is a distinct virus species belonging to serogroup IV. *Arch. Virol.* 143, 1637–1644.
- Kaloo, G., 1986. Tomato. In: *Breeding Habits Selection Bases and Breeding Methods*. Allied Publisher Private Limited.
- Kharkongar, H.P., Khanna, V.K., Tyagi, W., Rai, M., Meetei, N.T., 2013. Wide Hybridization and Embryo-Rescue for Crop Improvement in Solanum. *Agrotechnology* 11, 004. <http://dx.doi.org/10.4172/2168-9881.S11-004>.
- Krishnareddy, M., Usha-Rani, R., Kumar, A., Madhavireddy, K., Pappu, H.R., 2008. First report of Capsicum chlorosis virus (Genus Tospovirus) infecting chili pepper (*Capsicum annuum*) in India. *Plant Dis.* 92, 1469.
- Kunkalikar, S., Sudarsana, P., Rajagopalan, P., Zehr, U.B., Naidu, R.A., Kankanallu, R.S., 2007. First report of Capsicum chlorosis virus in tomato in India. *Plant Health Prog.* <http://dx.doi.org/10.1094/PHP-2007-1204-01-BR>.
- Kunkalikar, S.R., Sudarsana, P., Bhanupriya, M., Arun, P., Rajagopalan, A., Tsung-Chi, C., Shyi-Dong, Y., Rayapati, A.N., Usha, B.Z., Kankanallu, S.R., 2011. Importance and genetic diversity of vegetable-infecting Tospoviruses in India. *Phytopathology* 101 (3), 367–376.
- Le May, N., Bouloy, M., 2012. Antiviral escapes strategies developed by bunyaviruses pathogenic for humans. *Front. Biosci.* 1 (4), 1065–1077.
- Liu, R., Vaishnav, R.A., Roberts, A.M., Friedland, R.P., 2013. Humans have antibodies against a plant virus: evidence from tobacco mosaic virus. *PLoS ONE* 8 (4), e60621.
- Mandal, B., Jain, R.K., 2010. Can plant virus infect human being? *Indian J. Virol.* 21 (1), 92–93.
- Mandal, B., Jain, R.K., Krishnareddy, M., Krishna Kumar, N.K., Ravi, K.S., Pappu, H.R., 2012. Emerging problems of Tospoviruses (*Bunyaviridae*) and their management in the Indian Subcontinent. *Plant Dis.* 96 (4), 468–479.
- Mehdi, S., Sudhakar, D.W., 2008. Tomato breeding for resistance to Tomato spotted wilt virus (TSWV): an overview of conventional and molecular approaches. *Czech. J. Genet. Plant Breed* 44 (3), 83–92.
- Miller, F.W., Alfredsson, L., Costenbader, K.H., Kamen, D.L., Nelson, L.M., 2012. Epidemiology of environmental exposures and human autoimmune diseases: Findings from a National Institute of Environmental Health Sciences Expert Panel Workshop. *J. Autoimmun.* 39 (4), 259–271. <http://dx.doi.org/10.1016/j.jaut.2012.05.002>.
- Mir, M.A., Brown, B., Hjelle, B., Duran, W.A., Panganiban, A.T., 2006. Hantavirus N protein exhibits genus-specific recognition of the viral RNA panhandle. *J. Virol.* 80 (22), 11283–11292.
- Montero-Astua, M., Rotenberg, D., Leach-Kieffaber, A., Schneewis, B.A., Park, S., Park, J.K., German, T.L., Whitfield, A.E., 2014. Disruption of vector transmission by a plant-expressed viral glycoprotein. *Mol. Plant Microbe Interact.* 27 (3), 296–304.
- Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* 15, 472–497.
- Olszewska, W., Steward, M.W., 2003. The molecular basis of the antigenic cross-reactivity between measles and cowpea mosaic viruses. *Virology* 310, 183–189.
- Pappu, H.R., Jones, R.A.C., Jain, R.K., 2009. Global status of Tospovirus epidemics in diverse cropping systems: successes achieved and challenges ahead. *Virus Res.* 141, 219–236.
- Pico, B., Herraiz, J., Ruiz, J.J., Nuez, F., 2002. Widening the genetic basis of virus resistance in tomato. *Sci. Hortic.* 94, 73–89.
- Powers, K.M., Kay, D.M., Factor, S.A., Zabetian, C.P., Higgins, D.S., 2008. Combined effects of smoking, coffee, and NSAIDs on Parkinson's disease risk. *Movement Disord.* 23, 88–95.
- Poysa, V., 1990. The development of bridge lines for interspecific gene transfer between *Lycopersicon esculentum* and *L. peruvianum*. *Theor. Appl. Genet.* 79, 187–192.
- Prochaska, J.J., Hilton, J.F., 2012. Risk of cardiovascular serious adverse events associated with varenicline use for tobacco cessation: systematic review and meta-analysis. *BMJ* 344, e2856. <http://dx.doi.org/10.1136/bmj.e2856>.

- Quik, M., Perez, X.A., Bordia, T., 2012. Nicotine as a potential neuroprotective agent for Parkinson's disease. *Movement Disord.* 27, 947–957.
- Rae, C.S., Khor, I.W., Wang, Q., Destito, G., Gonzalez, M.J., 2005. Systemic trafficking of plant virus nanoparticles in mice via the oral route. *Virology* 343, 224–235.
- Rao, K.V., Kiranmayee, K., Pavan, U., Jaya, T., Rao, A.V., Sree, Sadanandam, A., 2005. Induction of multiple shoots from leaf segments, in vitro-flowering and fruiting of a dwarf tomato (*Lycopersicon esculentum*). *J. Plant Physiol.* 162, 959–962.
- Ravi, K.S., Kitkaru, A.S., Winter, S., 2006. Iris yellow spot virus in onions: a new Tospovirus record from India. *Plant Pathol.* 55, 288.
- Reddy, D.V.R., Ratna, A.S., Sudarshana, M.R., Kiran Kumar, I., 1992. Serological relationships and purification of bud necrosis virus, a Tospovirus occurring in peanut (*Arachis hypogaea* L.) In India. *Ann. Appl. Biol.* 120, 279–286.
- Ritz, B., Ascherio, A., Checkoway, H., Marder, K.S., Nelson, L.M., 2007. Pooled analysis of tobacco use and risk of Parkinson disease. *Arch. Neurol.* 64, 990–997.
- Satyanaryana, T., Gowda, S., Lakshminaryan Reddy, K., Metals, E., Dawson, W.O., Reddy, D.V.R., 1998. Peanut yellow spot virus is a member of a new serogroup of Tospovirus genus based on small (S) RNA sequence and organization. *Arch. Virol.* 143, 353–364.
- Segeren, M.I., Siqueira, W.J., Sondahl, M.R., Lourencao, A.L., Medina Filho, H.P., Nagai, H., 1993a. Tomato breeding: 2. Characterization of F1 and F2 hybrid progenies of *Lycopersicon esculentum* X *L. peruvianum* and screening for virus and insect resistance. *Rev. Bras. Genet.* 16 (3), 773–783.
- Segeren, M.I., Sondahl, M.R., Siqueira, W.J., Medina, F.H.P., Nagai, H., Lourencao, L., 1993b. Tomato breeding: embryo rescue of interspecific hybrids between *Lycopersicon esculentum* Mill. and *Lycopersicon peruvianum* (L.) Mill. *Rev. Bras. Genet.* 16, 367–380.
- Shriver, L.P., Koudelka, K.J., Manchester, M., 2009. Viral nanoparticles associate with regions of inflammation and blood brain barrier disruption during CNS infection. *J. Neuroimmunol.* 211, 66–72.
- Sopori, M., 2002. Effects of cigarette smoke on the immune system. *Nat. Rev. Immunol.* 2, 372–377.
- Stevens, M.R., Scott, S.J., Gergerich, R.C., 1992. Inheritance of a gene for resistance to Tomato spotted wilt virus (TSWV) from *Lycopersicon peruvianum*. *Euphytica* 59, 9–17.
- Stevens, M.R., Price, D.L., Memmott, F.D., Scott, J.W., Olson, S.M., 2007. Identification of markers linked to Sw-7 a new Tomato spotted wilt virus resistance gene, derived from *S. chilense*. In: Abstracts from the 2007 Tomato Breeders Roundtable. The Pennsylvania State University, Penn- Sylvania.
- Sun, H.J.S., Uchii, S.W., Ezura, H., 2006. A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. *Plant Cell Physiol.* 47, 426–431.
- Sundaraj, S., Srinivasan, R., Culbreath, A.K., Riley, D.G., Pappu, H.R., 2014. Host plant resistance against tomato spotted wilt virus in peanut (*Arachis hypogaea*) and its impact on susceptibility to the virus, virus population genetics, and vector feeding behavior and survival. *Phytopathology* 104 (2), 202–210.
- Thomas, B.R., Pratt, D., 1981. "Efficient hybridization between *Lycopersicon esculentum* and *L. peruvianum* via embryo callus. *Theor. Appl. Genet.* 59, 215–219.
- Zhang, T., Breitbart, M., Lee, W.H., Run, J.Q., Wei, C.L., 2006. RNA viral community in human faeces: prevalence of plant pathogenic viruses. *PLoS Biol.* 4 (1), e3. <http://dx.doi.org/10.1371/journal.pbio.0040003>.