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Effective Thermophilic Composting of Crop Residues for Inactivation of Tobacco Mosaic Virus

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Abstract: An effective thermophilic composting bioreactor, in which a homogenous distribution of temperature was maintained at 63-65°C by the addition of a bioavailable carbon and low mixing, was developed. The bioreactor operated on a mixture of tomato plant residues-wood shavings-municipal solid waste compost infected with tobacco mosaic virus (TMV). The initial C: N ratio and moisture content of the compost mixture were adjusted to 30:1 and 60%, respectively. The composting process was successful in destroying the tobacco mosaic virus. The results showed that the ability of the untreated virus (inoculum) to infect tobacco plants (150 LL L^{-1}) was much higher than its ability to infect tomato plants (22 LL L^{-1}). The TMV completely lost its ability to infect the leaves of susceptible hosts (tobacco and tomato plants) after 96 hrs of controlled thermophilic (63-65 ºC) composting (or 126 h from the start of the composting process). Semilog plots of the ratio of the infection ability of the surviving virus to that of the initial inoculum (as measured by the number of local lesions) were developed. The decimal reduction time (the time necessary to reduce the infection ability of TMV by 1-log or 90%) was found to be 62.4 and 109.7 hrs for tobacco and tomato plants, respectively. The relatively short time required for complete inactivation of TMV in this study was achieved as a result of the extension of the thermophilic stage and maintaining a constant high temperature with a uniform temperature distribution by the continuous addition of the proper amount of bioavailable carbon (used cooking oil) and low mixing.

Key words: TMV, compost, thermophilic, temperature, inactivation, tomato remains

INTRODUCTION

Greenhouse tomato production represented 58% of the greenhouse total vegetable production in Canada in 2000, with a production area of 1550 hectares, yielding 182,736 tonnes[1]. Typical vegetable greenhouse operations produce 40-60 tonnes of organic residues per hectare per year, as a result of trimming and harvesting the crop, which must be disposed of properly[2]. Tomato crop is susceptible to various types of insects and diseases under greenhouse environments where optimum conditions of most pathogens are met. The most common insects are aphids, whiteflies, leafminers, tomato pinworms, armyworms, tomato fruitworm and cabbage looper. Insects, in addition to the damage caused by feeding on the plant, can spread bacterial, fungal and viral tomato diseases and provide infestation sites for others[3,4]. The spore forming bacterium *Bacillus cereus*, the fungi *Pythium Fusarium*, *Cladosporium*, *Botrytis*, *Alternaria* and *Phytophthora* and the viruses *tomato yellow curl* and *tobacco mosaic* are among the most reported plant pathogens affecting tomato crops under greenhouse conditions[5]. However, viruses cause the most damaging tomato diseases and can reduce plant yield by 70-100%[6]. The most common sources of virus inoculum are the debris of infected plants which serve as reservoirs for virus transmission to healthy plants[7,8]. Therefore, improper disposal of these plant residues can contribute to recycling of plant pathogens[9].

Composting of greenhouse wastes has been considered by NSDAFF[10] and ODAF[2] as the preferred organic waste management method, especially for the destruction of plant pathogens. Composting is the aerobic biological decomposition of organic matter[11,12], whose end product (compost) can be used to restore and preserve the environment[13]. It converts the unstable organic materials into a more stable form that is safer to use for improving soil fertility, tilth and water holding capacity[14]. In addition, composting reduces the volume of organic material to be spread, improves its handling properties and reduces odor, flies and pathogens[15].

Most bacterial and fungal plant pathogens are sensitive to heat with the exception of spore forming *Bacillus cereus* and a few species of *Fusarium oxysporum*, which are considered heat resistant[16-18]. However, the tobacco mosaic virus (TMV) is considered the most heat resistant plant pathogen and it

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has been known to survive for more than 50 years in dried plant parts[7,8,18]. Therefore, a thermal process that is capable of destroying TMV will certainly destroy other plant pathogens.

The aim of this study was to evaluate the effectiveness of a controlled thermophilic composting process of TMV infected tomato plant residues in eradicating the virus. The specific objectives were to: (a) infect healthy tomato plants with TMV and identify the virus in infected plants using an ELISA test, (b) test the ability of the system to extend the thermophilic phase and maintain a constant temperature, (c) evaluate the success of the TMV inactivation process using an ELISA test, (d) test the ability of the inactivated TMV virus (compost) to infect susceptible host plants and (d) determine the virus survival rate and the time required for total inactivation.

MATERIALS AND METHODS

The composting system shown in Fig. 1 consisted of a frame, three bioreactors (each with a mixing unit and an air supply unit) and data acquisition system. The frame was made of aluminum sheets and angles (3.2 mm thick). It held the mixing motors, flow meters, air and exhaust gas manifolds, tubing and the thermocouple wires. A detailed description of the frame can be found in Alkoaik[19].

Each bioreactor (Fig. 2) was constructed of 520 mm long and 203 mm (ID) polyvinyl chloride (PVC) tube having a wall thickness of 5 mm. A removable circular Plexiglas plate of 203 mm diameter and 6 mm thickness was recessed and secured into one side of the cylinder by means of six stainless steel screws (6 mm). A rubber gasket lining (2.5 mm thick o-ring) was added to the inner side of the circular plate to keep it tight. There was a small circular window (64 mm in diameter) on the removable circular plate, which was closed with a rubber stopper (No.13) and used as a sampling port. A PVC plate of 203 mm diameter and 6 mm thickness was glued into the other side of the tube. Each reactor was fitted into an aluminum ring, which was fastened into the frame by means of four bolts (6 mm) and nuts. A removable 10.5 mm diameter solid stainless steel shaft (having 5 stainless steel collars in which five bolts of 69 mm length and 6 mm diameter each were mounted) was mounted on two bearings inside each bioreactor. The shaft was rotated (5.76 rpm) by a thermally protected electric motor (Model No. 127P1486/B, D. C., Sigma Instruments Inc., Braintree, Mass, USA). There were three holes at the bottom and one at the top of the bioreactor, which were drilled and threaded to take a 12 mm nylon hose barb. The holes at the bottom were connected to a manifold by 6.4 mm diameter Tygon tubing and used for aeration, whereas the one at the top was used for the exhaust gas. Air was supplied continuously to the bottom of the bioreactor from the laboratory air supply. It passed through a pressure regulator and a pressure gage (to maintain the pressure around 5 kPa) and then through a water bath (to humidify the inlet air to nearly 100% saturation) and finally through a flow meter (Model 32461-14, Cole-Parmer Instrument Company, Vernon Hills. Illinois. USA) capable of measuring a flow in the range of 0.0566-0.566 $m^3 h^{-1}$. Both circular plates were insulated with 38.1 mm thick Styrofoam layer, while the tube was insulated with 38.1-mm thick Fiberglass.

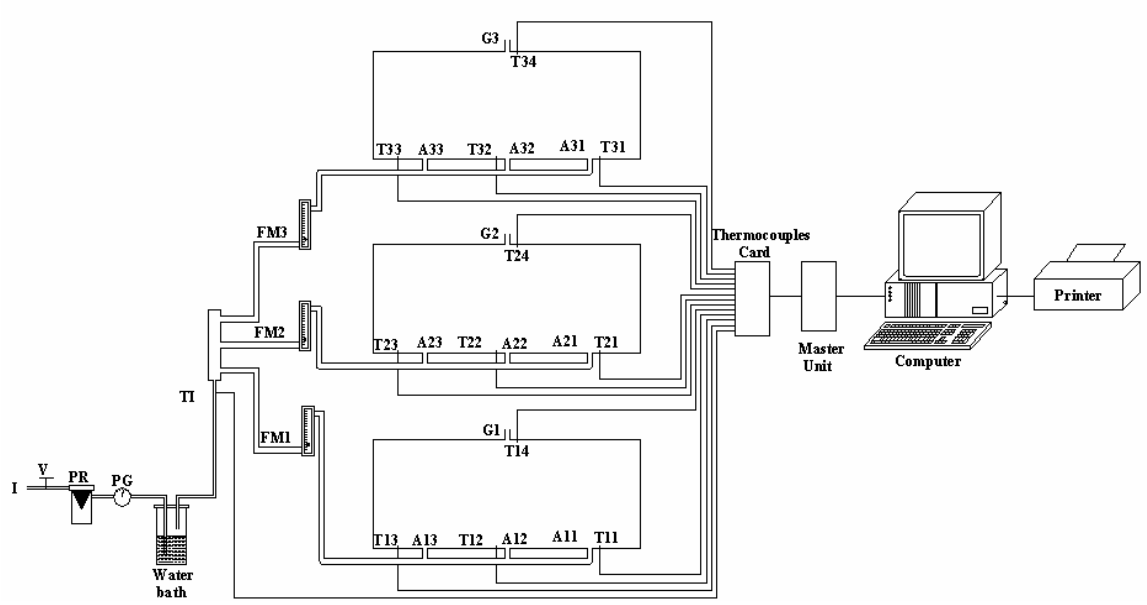
The data acquisition system consisted of a master unit (Multiscan 1200, Omega, Stamford, CT, USA), a thermocouple scanning card having 24 isolated differential input channels (MTC/24, Omega, Stamford, CT, USA), software, type T (copper-constantan) temperature sensors (Cole Parmer, Chicago, IL, USA), a personal computer (IBM Pentium IV) and a printer (Hewlett Packard Laser Jet 4) . The master unit was connected to a computer via RS 232 interface. Three thermocouples were located at the bottom of the bioreactor and were used to measure the temperature of the compost mass whereas the fourth was located at the top of the bioreactor, near the outlet air exit (21 mm away) and was used to measure the temperature of the exhaust gas. Thermocouple locations, on the bottom of all bioreactors, were chosen to be 65 mm from the inlet air.

Compost mixture preparation: The materials used in this study included tomato plant residues, wood shavings, municipal solid waste compost, urea and used cooking oil. Some characteristics of these materials are shown in Table 1. The tomato plant residues, wood shavings and municipal solid waste compost contained sufficient macro and micro nutrients. The used cooking oil and wood shavings had very high C: N ratios, while the tomato plant residues and municipal solid waste compost had low C: N ratios. The tomato plant residues (leaves, stems and some fruits) were collected from a greenhouse at an average moisture content of approximately 90 % and left over night at room temperature (≈ 25 °C) to partially dry (MC 76 %). They were then chopped into small pieces using a shredder (Model 242A645-515, 5HP, Briggs and Stratton, Plainfield, NJ, USA), mixed with wood shavings (1: 1.5 dry basis) and finally ground in a hammer mill (Model C-H, Horvick Manufacturing, NCC, Moorhead, Minnesota, USA) to an average size of 6.0 mm. A 15-day old municipal solid waste compost was added to the tomato trimmings mixture in order to introduce a wide range of active composting microorganisms. Urea [$CO(NH_2)_2$] was used as a nitrogen source (46% nitrogen) to adjust the C: N ratio to 30:1. Used cooking oil was used as a bioavailable carbon source. The heat balance performed on the system showed total heat losses of 51.2 kJ h^{-1} [19]. Since used cooking oil has an energy content of 36 kJ mL^{-1}, it was decided to add 18 mL every 12 hrs (1.46 mL h^{-1}) to compensate for the heat losses from the system, extend the length of the thermophilic stage and maintain the temperature at 63°C.

Table 1: Some characteristics of tomato plant residues, wood shaving and municipal solid waste compost

Characteristics	Tomato plant residues	Wood shavings	Municipal compost	Used cooking oil
Moisture Content (%)	76.0	8.0	58.6	NA
Total solids (TS) (mg g^{-1} DM)				
Volatile solids (VS) [1]	693.0	997.4	854.6	999.45
Ash	307.0	2.6	145.4	0.55
Nitrogen (mg g^{-1} DM)				
Total Kjeldahl Nitrogen	27.0	1.0	18.0	0.22
Ammonium Nitrogen	2.2	0.2	5.2	0.004
Carbon (mg g^{-1} DM)				
Total	327.0	499.0	440.0	775.0
Organic	260.0	390.0	350.0	--
Elemental Composition (mg g^{-1} DM)				
Ca	51.0	0.8	20.0	0.057
Na	0.7	0.0	6.2	0.301
Fe	0.4	0.0	2.8	0.14
Mg	4.7	0.1	1.8	0.008
Zn	0.0	0.0	0.1	ND
K	57.6	0.6	7.8	0.01
Cl	0.07	0.0	0.3	0.742
P	10.5	0.0	2.7	0.01
S	7.9	0.9	2.3	2.324
Others[2]	174.1	0.2	101.4	0.161
C: N	12.1: 1	499.0: 1	24.4: 1	3875.0: 1

These values are the average of three replicates (the coefficient of variation (CV) varied from 1.1 to 6.9%).
NA Not applicable
ND Not detectable
Volatile solids are the organic matter (largely carbon, oxygen and nitrogen) which are lost at 550ºC, leaving only the ash.
Others include mostly silica and other elements.



A-Inlet air ports, FM- Flow meter, G- Exhaust gas ports, I- Inlet air
PR- Pressure regulator, PG- Pressure gage, T- Thermocouple location, V- Valve

Fig. 1: In-vessel composting system

Infection of healthy tomato plants with virus: Tobacco mosaic virus (PV-1) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). In order to ensure that the TMV virus was active, one hundred and twenty, 4-week-old individually grown tomato plants (thirty each of Golden Boy, Roma, Red Robin and Scotia varieties) were infected with the virus. The plants were obtained from a specialized local nursery. Each plant was transferred into a large round plastic pot (12.5 cm) using Miracle-Gro-garden Soil for Vegetables as a potting media (Scotts Company, Marysville, OH, USA) and placed in a growth chamber. The tomato plants were irrigated weekly with a solution of Miracle-Gro (Scotts Company, Marysville, OH, USA) all Purpose water soluble plant fertilizer (Table 2) at the recommended rate of 4 mL L^{-1}.

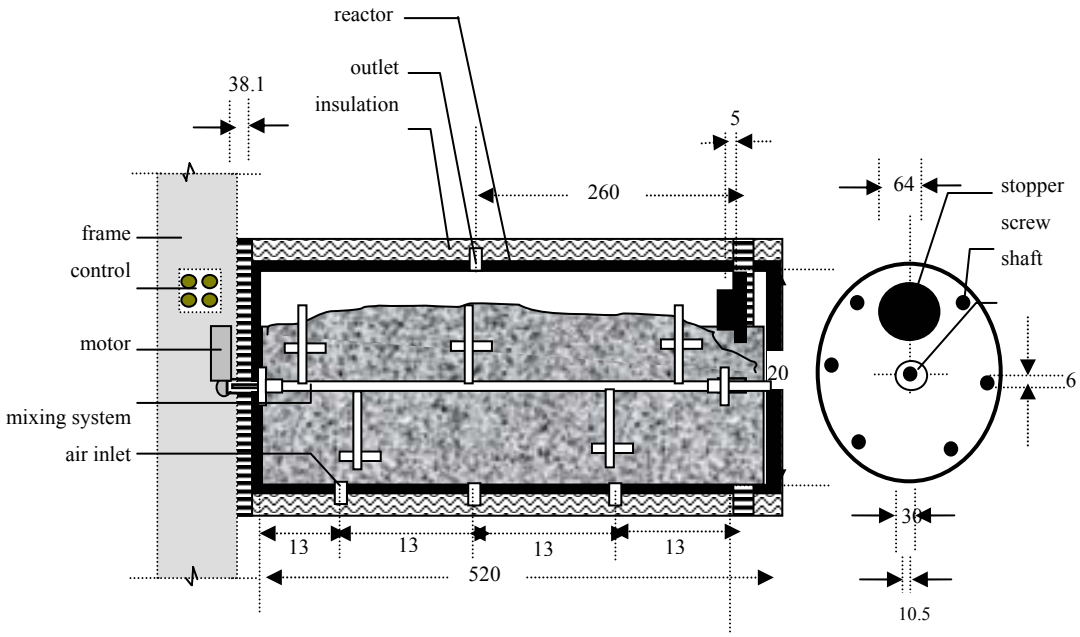


Fig. 2: Detailed schematic of the bioreactor

Table 2: Hydroponic fertilizer (Plant Products Co. Ltd., Brampton, Ontario, Canada)

Nutrient	Value
Macronutrient Content (%)	
N	10.0
P_2O_5	6.0
K_2O	16.0
Ca	7.2
Mg	2.2
S	2.9
Micronutrient Content (%)	
Fe	0.3000
Cu	0.0052
Mn	0.1042
Zn	0.0052
B	0.0157
Mo	0.0105

Tomato leaves were wetted and soil was soaked with the Miracle-Gro solution (~300 mL pot^{-1}). Fresh water was added when needed to compensate for the water lost by evaporation. The photoperiod was kept at 12 hr and the temperature was maintained in the range of 22 ± 2ºC. By the end of the first week of planting, tomato plants looked healthier and new leaves had developed.

The inoculation process illustrated in Fig. 3 was followed. The sealed TMV vial (contained freeze dried infected tissue) was carefully opened and the contents of the vial were placed in a pre-cooled (4 ºC) mortar. A volume of 9 mL of the inoculation buffer (0.1% peptone water, prepared by dissolving 1 g bacto-peptone in 1 L deionized distilled water and then autoclaving at 121 ºC and 103.4 kPa for 45 min) was added. A sterile pestle was used to thoroughly triturate the tissue. A few leaves on each young, healthy tomato plant were selected and marked. While one hand was used to support the leave to avoid over pressing, the other hand was used to mechanically damage some of the cells using abrasive cloth (240-J). Then, TMV inoculum was lightly rubbed onto the systemic host plants (tomato) using a sterile cotton swab. The infected tomato plants were labeled and left in their own individual containers under a 12 h light period in the growth chamber for the virus infection process to take place (incubation period).

Identification of virus in infected tomato plants: After ten days, the symptoms of infection (mosaic-like) started to develop. The symptoms are characterized by alternating patches of dark green (normal) and light green or yellowish colors on leaves. Dark green areas also appeared thicker and elevated giving the leaves a blister-like appearance[7]. The infected tomato plants were collected for virus identification according to the procedure illustrated in Fig. 4. Three infected tomato plants were selected at random and newly developed leaves (~1 g) were removed from each plant. Each sample was homogenized with the buffer solution in a mortar and then left aside (few min) to settle. The positive and negative controls, supplied with the ELISA kit, were prepared according to the manufacturer (Neogen Europe Ltd., Auchincruive, Scotland, UK) procedure and kept in a freezer (-10 ºC) for later analyses. Volumes of 100 µL of each sample and controls were transferred into a microtitre plate. After the addition of probe, antigen and color substrate, the plate was scanned using an ELISA plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Inoculum preparation: After confirmation of infection by TMV, a mixture of the infected plants (~200 g) was added to 500 g of the final compost mixture, throughly mixed and divided into small portions of 50 g each. Each portion was placed in a sachet (7 X 12 cm) made of porous fabric (muslin mesh). Each sachet was placed in a zip-lock bag and kept in a refrigerator at 4 ºC until required for the composting experiments.

Composting protocol: About 62 mL of used cooking oil was initially added to each 1.8 kg mixture of tomato residues: wood shavings: municipal solid compost (1:1.5: 0.28 ratio). Water was used to adjust the moisture content to 60%. The C: N ratio was adjusted to 30:1 by the addition of urea and the final mixture was mixed well. A single inoculum sachet of the plant pathogen, TMV was securely fixed to the mixing rod as shown in Fig. 5. The final compost mixture (3.5 kg) was then placed in the bioreactor. It occupied 75% of the total volume of the bioreacter (or 0.012 m^3). The plexiglas side wall was put in place and visual inspection of the bioreactor was done to detect any leakage before placing the insulation cover on the side wall. The mixing unit was started at 5 RPM and the system was operated at a constant aeration rate of 0.15 $m^3\ h^{-1}$ (0.17 volume per volume per min, vvm) during all experiments. The temperature was continuously monitored. Once the temperature peaked (after 31 h), a volume of 18 mL of the used cooking oil was added every 12 h in order to maintain the temperature above 55 ºC[19]. In order to maintain a constant temperature during the thermophilic phase, six bioreactors were used and samples were collected from each reactor at the end of the assigned treatment period to assess the inactivation of the TMV (24, 48, 72, 96, 120 and 144 hrs from the start of the thermophilic stage for reactors 1-6, respectively). The experiment was repeated 3 times.

Identification of virus in composting material: The sachets of TMV infected material were retrieved from the bioreactors at a pre-determined time (24, 48, 72, 96, 120 and 144 hrs after the peak temperature had been reached in reactors 1-6, respectively). An ELISA test was performed on the samples to determine the presence of TMV. A local lesion bioassay was then performed on two susceptible hosts (tomato and tobacco) to check the infectious ability of the TMV. The inoculation procedure previously described was followed. Once the infected plants exhibited the mosaic-like symptoms, the number of lesions was counted.

RESULTS AND DISCUSSION

Tomato infection process: *Tobacco mosaic virus* is transmitted principally by mechanical inoculation[20]. Different varieties (Golden boy, Roma, Red Robin and

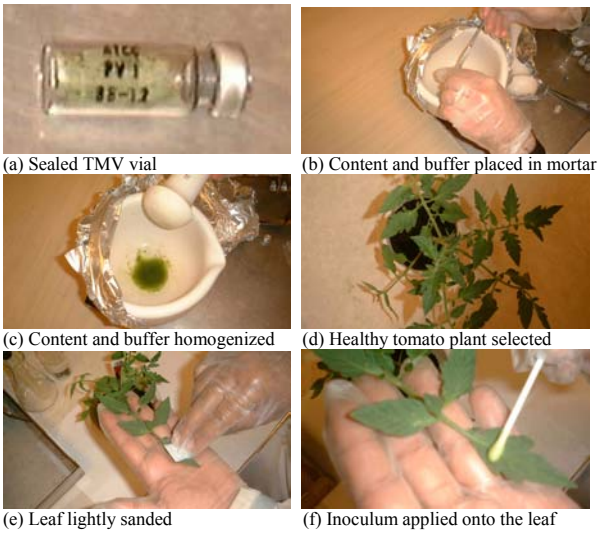


Fig. 3: The TMV inoculation procedure

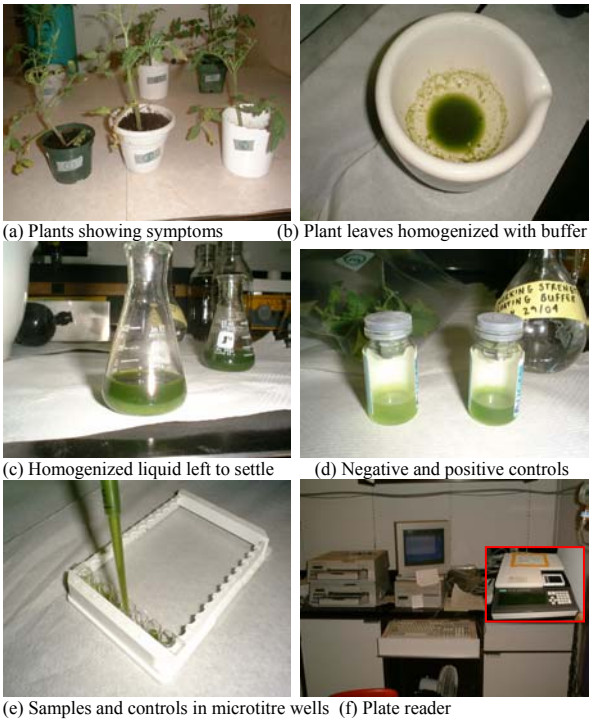


Fig. 4: ELISA test procedure

Scotia) of tomato plants were inoculated and the infected plants were used to determine the fate of TMV during the thermophilic composting process. The infected tomato plants started to develop the TMV symptoms after 7-10 days. Samples of newly developed leaves of the inoculated tomato plants were tested for TMV infection using ELISA kit. The ELISA results shown in Fig. 6 indicated that all tomato samples were infected with TMV.

Temperature: The temperature profiles of TMV experiments are presented in Fig. 7. During all

Fig. 5: Inoculum sachet position on the mixing shaft

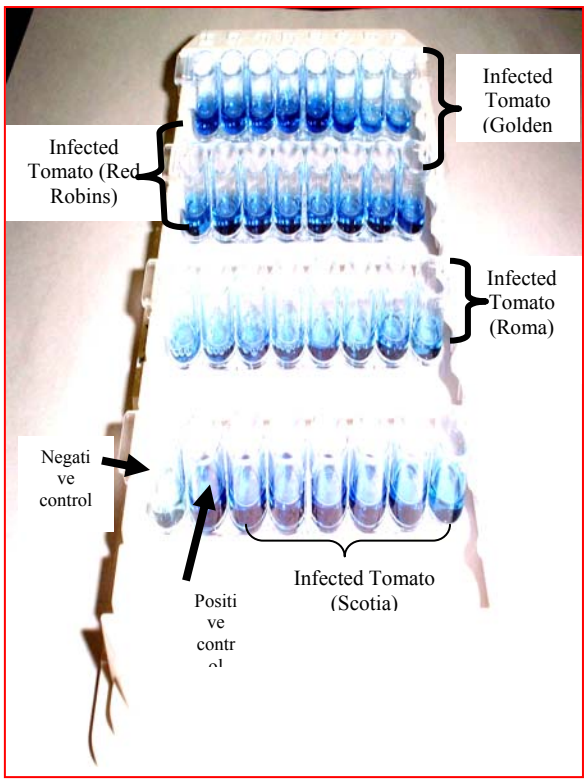


Fig. 6: ELISA test results of the infected samples

experiments, the average reactor temperature increased gradually reaching its peak after 31 hrs of operation. Thermophilic temperatures in the range of 63.2 – 64.9°C were maintained in all reactors (63.3 ºC ± 0.92 %, 63.2 ºC ± 0.96 %, 64.5 ºC ± 2.0 %, 64.8 ºC ± 1.9 %, 64.9 ºC ± 1.7 % and 64.9 ºC ± 1.7 % for a duration of 24, 48, 72, 96, 120 and 144 hrs in the first, second, third, fourth, fifth and sixth reactors, respectively). These temperatures were maintained by the addition of used cooking oil as a bioavailable carbon source which compensated for the total energy loss from the system.

Assessment of virus inactivation: The retrieved samples were tested for the ability of the TMV virus to infect susceptible host plants (tobacco and tomato) using a local lesion bioassay. The local lesion bioassay performed on tobacco and tomato plants showed an initial concentration (infected, untreated material) of 150 and 22 local lesions/leaf (LL L^{-1}), respectively. These were reduced to 35, 12, 2, 0, 0 and 0 LL L^{-1} for

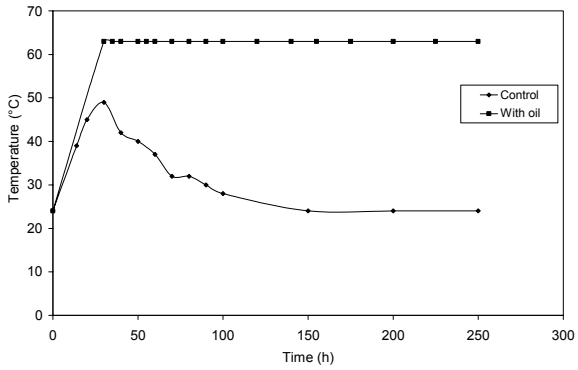
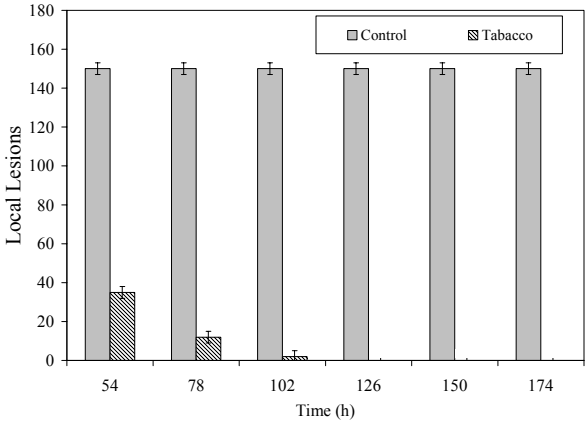
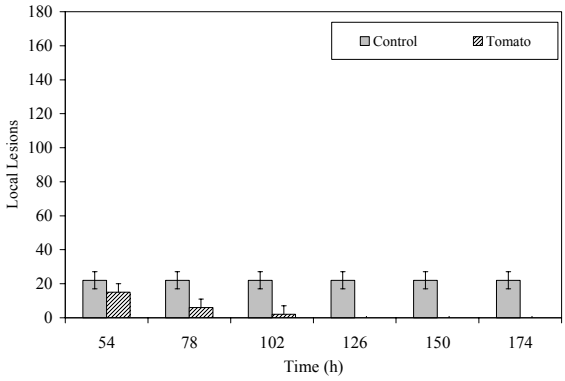


Fig. 7: Temperature profile for composting material



(a). Tobacco



(b). Tomato

Fig. 8: TMV local lesions count

the tobacco plants and 15, 6, 2, 0, 0 and 0 LL L^{-1} for the tomato plants after 24, 48, 72, 96, 120 and 144 hrs (from the time of temperature peak) of thermophilic treatment (63.2 – 64.9°C). The results indicated that TMV completely lost its ability to infect the leaves of the two hosts after 4 days of thermophilic composting (Fig. 8).

Ryckeboer *et al.*[21] investigated the fate of *tobacco mosaic virus* (TMV) during composting and found that TMV was significantly reduced (by a factor of three) after 19 days of composting at 58 ºC and was reduced even further when the composting treatment was operated at 68 ºC for 12 days.

Bollen and Volker[22] stated TMV is not completely inactivated in some composting systems and can be directly transmitted to plant roots without a vector. In the current study, TMV completely lost its ability to infect the leaves of a susceptible host after less than 4 days of controlled thermophilic composting and the compost material produced from this system is considered safe for land application. It should be noted that the controlled composting process has other advantageous destruction mechanisms beside high temperature such as microbial competition (antagonism) and toxicity of by-products (pH, ammonia and organic acids) released as a result of plant residues decomposition that could inactivate TMV in a shorter time as reported by Lopez-Real and Foster[16] and Bollen[18].

According to Hogland *et al*.[23] and Ryckeboer *et al*.[21], the high temperature (about 60 ºC) that can be reached during the thermophilic stage of composting is the most important factor for inactivation of plant pathogens. However, temperature fluctuations, clumping of solids and improper mixing of raw material and amendment are the most negative characteristics of solid mixtures during composting process[11]. The inactivation period of TMV observed in the current study is significantly shorter than the two weeks reported by Marciniszyn and Gottschall[24], the 21 days reported by Noble and Roberts[25] and the 12 days at 68 ºC reported by Ryckeboer *et al*.[21]. It was achieved as a result of extending the thermophilic phase and maintaining a constant high temperature in the bioreactor by the continuous addition of bioavailable source of carbon (used cooking oil) and the utilization of a continuous low mixing (5 RPM) that eliminated any temperature gradient in the composting system.

The relationship between number of local lesions (caused by the surviving virus) and composting time can be expressed by survival curves as shown in Fig. 9. Survival curves are semilog plots of the ratio of the concentration of surviving organisms to the initial number versus time. They are commonly used to interpret the inactivation kinetics of microorganisms. The destruction/inactivation of the TMV virus during the composting process can be described with the following first order equation:

$$\frac{dN}{dt} = -kN \qquad (1)$$

Where:

N is the initial number of local lesions
t is the composting time (h)
k is the decay constant (h^{-1})

The ability of the TMV virus to infect susceptible hosts was tested using healthy tobacco and tomato plants. Although identical samples were used in both tests, the number of local lesions observed on the infected tobacco plants by the TMV virus from the untreated sample (initial) was much higher (150) then

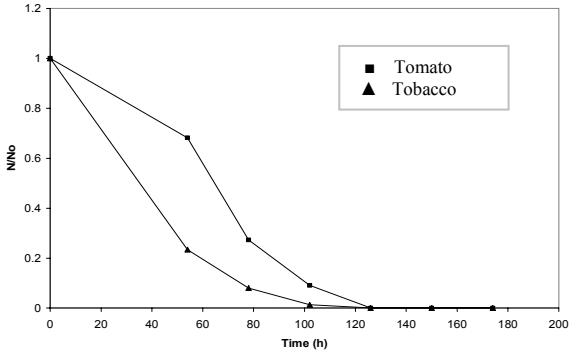


Fig. 9: Survival curves for TMV

the number of local lesions observed on the tomato plants infected with similar untreated sample. Therefore, equations 2 and 3 can be used to describe the ability of the TMV virus to infect different, susceptible hosts (tobacco and tomato) as a function of composting time and initial concentration.

$$\frac{N}{No} = e^{-0.0369t} (R^2 = 0.93) \text{ for tobacco} \qquad (2)$$

$$\frac{N}{No} = e^{-0.021t} (R^2 = 0.94) \text{ for tomato} \qquad (3)$$

From these equations, the decimal reduction time, which is defined in this study as the time necessary to reduce the infection ability of TMV by 1-log or 90% at a given temperature was found to be equal to 62.40 and 109.65 hrs for tobacco and tomato plants, respectively.

CONCLUSION

The results showed that the ability of the untreated virus (inoculum) to infect tobacco plants (150 LL L^{-1}) was much higher than its ability to infect tomato plants (22 LL L^{-1}). The controlled thermophilic composting process was successful in inactivating the *tobacco mosaic virus*. The TMV virus completely lost its ability to infect the leaves of susceptible hosts (tobacco and tomato plants) after less than four days of controlled thermophilic (63-65 ºC) composting (or 126 h from the start of the composting process). Semilog plots of the ratio of the infection ability of the surviving virus to that of the initial inoculum (as measured by the number of local lesions) were developed. The decimal reduction time (the time necessary to reduce the infection ability of TMV by 1-log or 90%) was found to be 62.4 and 109.7 hrs for tobacco and tomato plants, respectively. The relatively short time required for complete inactivation of TMV in this study was achieved as a result of extending the thermophilic stage and maintaining a constant high temperature with a uniform temperature distribution by the continuous addition of the proper amount of bioavailable carbon (used cooking oil) and low mixing.

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