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Evaluation of inoculum density of different postharvest pathogens on infection of tomato fruit

NIREN MAJUMDAR*and NAKUL CHANDRA MANDAL

Department of Plant Protection, Palli-Siksha Bhavana (Institute of Agriculture), Visva-Bharati, Sriniketan, Birbhum, West Bengal, India

***E-mail:** niren_majumder@yahoo.com

ABSTRACT: Tomato at its postharvest stage is affected by plethora of pathogens of which *Aspergillus niger*, *Rhizopus stol onifer* and *Fusarium* spp are most important. The initial inoculm density on host surface is critical to successful infection leading to wastage of product and may require culling within the package resulting considerable economic loss. The critical role of inoculum density of these three postharvest pathogens under *in vitro* condition has been determined. Critical threshold limits are linked to incubation period, generally at higher density, incubation period is reduced. While no visible symptom is discernible before 48 hours of incubation for *Aspergillus niger* and *Fusrium oxysporum* but becomes evident with *Rhizopus stolonifer* at 24 hours of incubation. The critical threshold limit determined for *Aspergillus niger* was 10^1 after 72 hours but higher inoculum density of 10^4 for 48 hours of incubation. Similarly, for *Fusrium oxysporum* the value is peggedat 10^3 during 48 hours which is not reduced at higher inoculums. In case of *Rhizopus stolonifer,* a fast growing fungus requires only 24 hours time for the inoculums density of 10^4. Faster rate of rotting was noticed at conc. of 108 for *Aspergillus niger,* 10^8 for *Fusrium oxysporum* and 10^2 for *Rhizopus stolonifer.*

Keywords: Inoculum, *Rhizopus stolonifer*, *Aspergillus niger*, *Fusarium oxysporum*, postharvest, tomato

INTRODUCTION

Tomato is a vitamin - C rich vegetable grown through out the world and consumed as raw and processed products as well as a daily ingredient of the kitchen. In India it is available year the round either as fresh or stored product, sometimes requires long distance transport between the production site and wholesale and retail markets. It is one of the most favourite fruit vegetables in the world with availablity throughout the seasons. The crop has been affected by various pre- and postharvest diseases. Large numbers of postharvest pathogens attack fruits on transit and thus reduces the quality and quantity having consequences on economic losses both to growers and consumers. Postharvest diseases account for about 50 % of losses in fruits stored in poor conditions especially under high humidity (Agrios, 2005; FAO, 2002) and found to be extreme in poor nations having inadequate infrastructure, processing and transport, The most important fungi causing post-harvest diseases include *Penicillium* spp., *Aspergillus* spp., *Alternaria* spp., *Botrytis cinerea*, *Monilinia lax*, and *Rhizopus stolonifer*. Arinze ,1986 reported that association of the fungi *Rhizopus stolonifer* (Ehrenb.) Lind, *Fusarium oxysporum* (Mart.) Sacc., *F.solani* Schlecht, *Aspergillus niger* van Tiegh., Penicillum sp. and *Lasiodiplodia theobromae* Pat. with the post-harvest disease of tomato fruits in Southern Nigeria. Physical injury or physiological breakdown of the commodity aggravate the problem by most microorganisms. *Fusarium* rot on tomato fruits are often caused by different *Fusarium* species (Denis 1983; Mehrotra 1989; Sherf and Macnab 1986; Solunkhe and Desai 1984)The succulent epicarp which enable the fungal hyphae of *Fusarium* spp. to penetrate deeply into the fruit (Tournas & Katsoudas 2005) may extend into the centre. The consumers preference of fresh and healthy (Nurulhuda *et al*. 2009) fruits make the diseased units unmarketable.

In the retail market of NewYork, USA losses was as high as 80% due to major pathogens like *A. alternata* and *R. stolonifer*. (Ceponis and Butterfield 1979). Abdel Mallek *et al*. (1995) pointed out that *R. stolonifer* from diseased tomatoes was isolated in a frequency of 35.9% only overcame by *A. alternata* in a frequency of 57.7%. In other studies, a suvey conducted in 15 different local markets, in twostate of Morelos, Mexico showed *R. stolonifer* is the most predominant fungus affecting almost 50% of the samples (unpublished data). In general, pathogenicity of *R. stolonifer* on tomato is not associated with strain, spore load, nutrient status or stage of maturity of tomato but even at the lowest concentration of 10^1 in mature and mature-green tomatoes (Silvia Bautista et al.2008) The international importance of *Rhizopus*

stolonifer is reflected in Snowdon, (1990) Besides Fusarium spp, Ebele, (2011) reported the importance of *Aspergillus niger.*

Simlarly other fungi reported on tomato include *Aspeergillus flavus, Fusarium oxysporum, Monilocaetes infuscaus*, *Penicillum* spp, *Certolystis finbriata*, *Diapoc batatalis* (Snowdon , 1990) include Dasgupta and Mandal 1986) The lesion length on both green and mature fruit due to *R.stolinifer* depends upon spore concentrations, which is positively correlated. Lesion length may not be directly related to disease index and the present study was conducted. *Aspergillus* sp, *Rhizopus* sp and *Fusarium* sp. are few of the most prevailing postharvest pathogens of tomato.

MATERIALS AND METHODS

The fruit

Freshly harvested tomato fruits (variety –NS4032) were collected from the local market with uniform, size, shape and maturity. The fruits were cleaned with tap water followed by surface sterilized with 0.1% Mercuric chloride for 1 minute and subsequently washed three times with sterile distilled water and extra water on surface was removed by sterile absorbent cotton.

The Pathogens

Three pathogens namely *Aspergillus niger, Rhizopus stolonifer* and *Fusarium oxysporum* were isolated on PDA from the naturally rotten fruit. Identified pathogens as pure cultures were obtained through single spore culture technique. These pathogens were maintained on PDA as pure culture in slants at 4ºC for experimentation. Ten days old culture was used.

Preparation of spore suspension

Fungal spores used for these experiments were collected from 10 days old cultures of fungi grown on the PDA media as described above. Mycelial mats containing spores was taken within a culture tube containing sterile distilled water and thoroughly shaken followed by a passage through a glass wool filter to remove hyphae. Different spore concentrations (10^1 to 10^8 spores / ml) were obtained by serial dilution method after obtaining a mother spore suspension standardized with haemacyto meter. Fresh spores were used for inoculation.

Inoculation and incubation of tomato

Surface sterilized apparently healthy tomato fruits were artificially injured with the help of sand paper by rubbing in the equatorial region of the fruit with a size of about 10mm. Spore suspension of different concentrations were taken in separate sterile syringes and individual fruits were inoculated by placing two drops of spore suspension on the injured site. Immediately after inoculation fruits were placed within a sterile polypropylene bag along with sterile cotton swab to maintain the high humidity. All activities were conducted in a laminar flow chamber to avoid contamination. Inoculated fruits were incubated at 28 ±1ºC for up to 10 days. Ten fruits were taken for each treatment with three replications.Treatments were made as follows

T_1 = Control without injury, T_2= Control with injury, T_3= Spore concentration of 10^1, T_4= Spore concentration of 10^2, T_5=Spore concentration of 10^3, T_6=Spore concentration of 10^4 ,T_7=Spore concentration of 10^5, T_8=Spore concentration of 10^6 , T_9=Spore concentration of 10^7 , T_{10}=Spore concentration of 10^8

PDI (Percent Disease Index) calculation

0-9 scale of disease have been adopted for calculating the PDI . The scale is as follows

Disease Grade	Percent of fruit surface infection
0	: No symptom/infection
1	: >5 % infection
2	:5-10% of infection
3	:10-15% of infection
4	:15-25% of infection
5	:25-40% of infection
6	:40-60% of infection
7	:60-80% of infection
8	:80-99% of infection
9	:100% of infection

$$PDI = \frac{\sum Alldiseaseratings}{NoofobservationXMaximumdiseasegrade} X100$$

Statistical analysis

Statistical analysis of the data done by CRD using ANOVA in the MS excel sheet.

RESULTS

Table 1 shows that the PDI values differed among treatments however, in the early phase of incubation the critical threshold limit was obtained at treatment T6. In certain treatments

PDI were abnormally high and it may be possible that pathogen progressed internally without external manifestation of infection. The initial variation in host resistance cannot be ruled out. The rate of progress of infection in relation to incubation period is also not uniform when data on 4days and 5days of incubation is compared. Uninjured fruits remain unaffected throughout the period of experiment but injured uninoculated fruit showed first infection only on 6th day but attained only 51.85% on 9th day while the injured and inoculated fruit 100% PDI reached. After 06 days of incubation there are little variation in PDI among treatments. Data suggests that for quick varietal screening higher concentration of spores either to opt for 10^8 spores or one more day of incubation.

Table 1. Progress of infection (PDI) of *A.niger* under different inoculums densities

Treatment	48 hrs	72 hrs	96 hrs	120 hrs	144 hrs	Mean
T_1	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
T_2	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	11.11 (19.47)	2.22 (3.89)
	0.00 (0.00)	31.04 (33.73)	44.44 (41.80)	70.37 (57.02)	88.89 (70.53)	46.95 (40.62)
T_4	0.00 (0.00)	22.22 (28.12)	40.74 (39.63)	70.37 (57.14)	96.30 (81.04)	45.93 (41.19)
T_5	0.00 (0.00)	18.51 (25.43)	44.44 (41.81)	70.37 (57.02)	88.89 (70.53)	44.44 (38.96)
T_6	7.41 (15.80)	33.33 (35.06)	51.85 (46.09)	74.07 (59.43)	88.89 (74.14)	51.11 (46.10)
T_7	7.41 (15.80)	22.22 (28.12)	40.74 (39.66)	69.24 (56.33)	88.89 (74.20)	46.44 (42.82)
T_8	18.52 (25.43)	42.15 (40.48)	55.55 (48.21)	70.37 (57.02)	92.59 (77.19)	55.84 (49.67)
T_9	22.22 (28.12)	45.55 (42.45)	62.96 (52.57)	74.07 (60.02)	92.59 (77.02)	59.48 (52.04)
T_{10}	33.33 (35.26)	49.26 (44.58)	62.96 (52.65)	81.48 (64.81)	100.00 (90.00)	65.41 (57.46)
SEm (±)	0.45	1.66	2.11	2.21	4.22	
CD (p=0.05)	1.33	4.90	6.22	6.53	12.45	

No visible infection detected after 24 hours of incubation; @ infection not due to *A. niger*

Table 2. Progress of infection (PDI) of *R.stolonifer* under different inoculums densities

Treatment	24 hrs	48 hrs	72 hrs	96 hrs	Mean
T_1	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
T_2	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
T_3	0.00 (0.00)	55.56 (48.25)	70.37 (57.02)	88.89 (70.53)	53.71 (43.95)
T_4	0.00 (0.00)	59.26 (50.35)	74.07 (59.43)	92.59 (77.03)	56.48 (46.70)
T_5	0.00 (0.00)	62.96 (52.57)	7037 (57.02)	88.89 (70.54)	55.56 (45.03)
T_6	3.70 (11.09)	51.58 (45.91)	70.37 (57.14)	92.59 (77.19)	54.56 (47.83)
T_7	3.70 (11.09)	55.56 (48.21)	70.37 (57.05)	77.78 (62.09)	51.85 (44.61)
T_8	7.41 (15.80)	59.26 (50.34)	74.07 (59.43)	85.19 (67.75)	56.48 (48.33)
T_9	14.81 (22.35)	51.85 (46.06)	67.27 (55.12)	85.19 (67.37)	54.78 (47.72)
T_{10}	14.81 (22.63)	59.26 (50.34)	70.37 (57.02)	96.30 (81.04)	60.19 (52.76)
SEm (±)	0.91	1.68	1.18	3.59	
CD (p=0.05)	2.69	4.97	3.48	10.60	

From the table it is clear that the symptom development on tomato was faster as compare to the *A.niger* and *F.oxysporum*. Symptom started showing rotting on very next after inoculation of fruit. As the concentration increase the disease severity also increase. On third day itself *Rhizopus stolonifer* causes rotting of about 60% in all the treatment except T_1 and T_2 where no infection was observed till 7 Day where all the treated fruit rotten 100%. It was clear that the *Rhizopus* causes quick rotting as compare to *Aspergillus niger* and *Fusarium oxysporum*. In treatment T_3,T_4,T_5 there were no symptom on day2 but on third day more than 50% rotting symptom were observed which indicate there may be internal progression of disease which has not been expressed but on day3 sudden rotting were observed. On day6 all the fruits in all the treatment were rotten almost 100% except T_1 andT_2. T_6 on word the symptom develop on day2.

R. stolonifer has a radial nature of growth both internally and externally therefore quick damage become possible. *R.stolonifer* at higher concentration T_8,T_9 and T_{10} become self inhibitory in germination of spores therefore rate of progress perhaps were lower. Gene resistance in fruit to be checked at of higher inoculum dose of 10^7 and 10^8 after aperiod of 96 hrs of incubation in *A.niger*.

Table 3. Progress of infection (PDI) of *F. oxysporum* under different inoculum densities

Treatment	72 hrs	120 hrs	168 hrs	216 hrs	Mean
T_1	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
T_2	0.00 (0.00)	0.00 (0.00)	29.63 (32.88)	100.00 (90.00)	32.41 (30.72)
T_3	0.00 (0.00)	11.11 (19.47)	40.74 (39.63)	81.48 (64.81)	33.33 (30.98)
T_4	3.70 (11.09)	33.33 (35.26)	48.15 (43.94)	88.89 (70.57)	43.52 (40.22)
T_5	7.41 (15.80)	29.63 (32.98)	51.85 (46.06)	70.37 (57.14)	39.82 (37.99)
T_6	3.70 (11.09)	25.93 (30.51)	29.63 (32.88)	51.85 (46.06)	27.78 (30.14)
T_7	14.81 (22.35)	29.63 (32.95)	51.85 (46.12)	63.89 (53.18)	40.05 (38.65)
T_8	14.81 (22.63)	33.33 (35.24)	51.85 (46.06)	81.48 (64.59)	45.37 (42.13)
T_9	29.63 (32.95)	66.67 (54.81)	77.78 (62.02)	100.00 (90.00)	68.52 (59.94)
T_{10}	51.85 (46.07)	81.48 (64.59)	96.30 (78.93)	100.00 (90.00)	82.41 (69.90)
SEm (±)	1.27	1.33	2.04	1.77	
CD (p=0.05)	3.75	3.91	6.01	5.21	

No visible infection detected after 24 hours of incubation; @ infection not due to *F. oxysporum*

The table-3 shows that cottony leak of tomato due to *Fusarium oxysporum* causing 100% rot within 168 hours of incubation for the inoculum density 10^8. Inoculum density at Treatment T_4 and higher concentrations results in development of the symptom & rotting even on day 3 of incubation. In treatment T_2 (control with injury but without inoculm) other type of symptom & rotting was different as that of *Fusarium* rot and the source of infection may be by other fungi or bacteria of latent or quiescent nature. Either Day 7 or Day 8 should be considered as optimum incubation period as around 50% rotting of fruits are noticed. In higher inoculums density of treatment T_{10} more than 96% rot became evident at 144 hours of incubation. The minimum titrable inoculum density concentration thus becomes 10^2 at T_4.

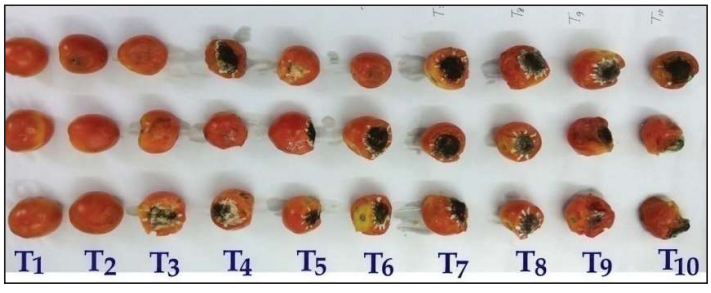

Plate.1 Effect of spore density of *Aspergillus niger* on infection of tomato fruit

DISCUSSION

Fungal spoilage of tomatoes is attributable to the high water content, environmental conditions, state of handling, state of storage facilities, the fungal load of the handlers and the quality of the tomatoes.Onuorah Samuel and Orji(2015) isolated different postharvest fungi of tomato from Awka market,Nigeria with inoculum density ranges from 1.3 x 10^3 to 2.0 x 10^3 cfu/ml. The fungal isolated from the fruits were *Aspergillus niger, Rhizopus stolonifer, Fusarium oxysporum, Saccharomyces cerevisiae, Alternaria alternata, Penicillium digitatum* and *G.eotrichum candidum*

It was evident that 10^6conidia per ml could serve as the inoculum potential of *A. alternata* (Verma, 2004). It is also to be noted that dispatch time should be shorter so that the cullage loss is reduced and this can be achieved at whole seller point with improvement in the technique of determination of pathogen load. Spore concentration of 10^6 spores/ml produced the most prominent disease development by *Colletotrichum gloeosporoides* in rubber tree (*Hevea brasiliensis*) (Sangeethasiva & Sepiah, 2016) our study suggest that increase in spores concentrations quicker the disease development by *A.niger, R.stolonifer* and *F.oxysporum*. on tomato. Minimum Spore concentration of 10^4 per ml develop the typical disease symptom 48 and 24 hrs after incubation by *A.niger* and *R.stolonifer* respectively at ambient room temperature where as spore density of 10^2 was sufficient for disease development in case of *F.oxysporum* at 24 hrs incubation. Kakvan *et al*. (2012) evaluated that pathogenicity of *Alternaria* isolates was capable of causing infection on citrus leaves from suspension of conidia (10^5 conidia /ml) prepared from 5-7 days old PDA cultivated isolates. Yousefi & Hagian Shahri (2009) conducted pathogenicity tests by inoculating slightly wounded plant tissue with conidial suspension adjusted to 1.5×10^4 conidia per ml of *Alternaria altenata* using a hemocyto meter that was capable of causing infection within two to four days. It was reported that inoculum concentration of 10^6 spores/ml was the optimum concentration to cause disease development on leaves (Bertetti et al., 2009; Dillard, 1989; Forcelini, 2013; Makowski, 1993). Kumar and Rao, 1979 reported that minimum spore concentration 400000 per ml is essential for disease establishment on wheat leaf with 5 week old plant by *Alternaria triticina*. Disease severity increased with inoculum density of *Phytophthora capsici* on different isolates and cultivers of pumpkin and pepper (Byung et al, 2001)

CONCLUSION

Increase in spore concentration resulted in proportional increase in disease incidence, implying the importance of spore load in storage facilities to the disease incidence. *R.stolonifer* at higher concentration T_8,T_9 and T_{10} become self inhibitory in germination of spores therefore rate of progress perhaps were lower. *Fusarium oxysporum* takes longer time to complete rotting of tomato as compare to *Aspergillus niger* and *Rhizopus stolonifer* to tomato.

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