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Survival and Management of onion soft rot caused by *Erwinia carotovora* var. *carotovora*

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Abstract: **Studies on the nature and extent of bacterial inoculum in storage godowns of onion revealed the occurrence of *E. carotovora* var *carotovora* in rootlets, gunny bags, godown sweeping and leaf debris and infected bulb in the field which may serve as source of inoculum. *E. carotovora* var *carotovora* survived better in the combination of sterilized soil + leaves + bulbs, which retained more number of bacteria even after six months of inoculation. Half of the inoculum load was detected in the unsterilized soil + leaves + bulbs. Studies on the detection of seed-borne nature of bacterium revealed that bacterial oozing from seed contains *E. carotovora* var. *carotovora*. Polyclonal antibodies were raised against unfractionated protein of *E. carotovora* var. *carotovora*. Indirect ELISA was carried out to detect the antigen and it was found that 1:1000 dilution of antibodies was necessary to detect the antigen. Seed treatment with *Pseudomonas fluorescens* FP7 was effective in checking the seed-borne nature of *E. carotovora* var. *carotovora* besides increasing vigour index of seedlings. Formalin at two per cent concentration also checked the growth of *E. carotovora* var. *carotovora*.**

***Key Words:** Erwinia carotovora var. carotovora, onion, serodiagnosis, biocontrol, Pseudomonas fluorescens.*

Introduction

India is the second largest producer of onion in the world followed by China. In India, the area under onion was 3.22 lakh ha with a production of 45.5 lakh tonnes during the year 1996-98 (Anon, 2000). Nearly 40 per cent of the production is lost during postharvest handling and due to sprouting, of which microbial spoilage alone constituted 15-20 per cent of the total loss (Pantastico and Banstista, 1976; Bhagchandani *et al.*, 1980). Soil-borne populations of soft rot *Erwinia* have been implicated circumstantially as the primary source of inoculum in storage. The present investigations were taken up to detect the presence of primary source of inoculum, survival of pathogen and management with chemical, physical and biocontrol agents.

Materials and Methods

Source of inoculum from seed

Seed health testing for bacterial infection was carried out using blotter paper technique and roll towel method. Twenty five seeds were taken at random and placed at equidistance without surface sterilization and with surface sterilization (0.1% $HgCl_2$) on three layered sterile moisture blotters in petri plates. Each treatment was replicated four times. The plates were incubated at alternating UV light and dark for 12 h at 20 ± 2°C for seven days. The seeds were examined under a stereomicroscope for identification of pathogens. The number of infected seeds were counted and expressed as percentage and bacteria was isolated and purified for identification purpose (ISTA, 1993).

Production of Polyclonal Antibodies and Indirect ELISA

Antigen preparation

Log-phase cultures of bacteria were centrifuged at 13,000g for 5 min. Pellets were washed twice with 0.01M phosphate buffer

Table 1. Source of inoculum for postharvest spoilage of onion bulbs

Sl.No.	Part examined	Soft rot bacteria	Fungal pathogen
i	Godown sweeping	+++	++
2	Soil	+++	++
3	Leaves	+	-
4	Scales	-	++
5	Root lets	++	+
6	Gunny bags	+++	+

- No infection + Stray infection ++ Mild infection +++ Severe infection

saline (PBS, pH 7.4) at 40°C. Then it was resuspended in the same buffer for further preparation as required. The supernatant was collected and dialyzed with 0.01 M phosphate buffer (pH 7.0) overnight at 4°C to remove the salts. Protein estimation was done according to Bradford (1976). The dialyzed extract was used as antigen to raise antibodies against the unfractionated protein, after adjusting the protein concentration to 100 µg/ml.

Raising of Polyclonal Antibodies (PAbs)

Polyclonal antisera (PCA) against extracellular proteins of *E. carotovora* var. *carotovora* (40 kDa polypeptide) were raised by the intramuscular immunization method in rabbit as described by Shanmugam *et al.* (2002) with slight modification. Adult New Zealand white rabbits weighing about 1.5 kg (Courtesy: Pasteur Institute, Coonoor, India) were used. One milliliter of antigen was mixed with 1 ml of Freund's complete adjuvant and emulsified in a cyclomixer. The emulsion was administered intramuscularly in rabbit using sterile syringe with 22G needle. After the first immunization, three injections were given at 10 day intervals by mixing with Freund's incomplete adjuvant. Immune bleeds were taken from the marginal ear vein 10 days after the last injection, followed by another bleeding after a week. The serum was collected in sterile glass vials and kept for one hour at room temperature at slanting position for retraction of the clot. Then it was kept at 4°C in the same position overnight. The serum was then separated and centrifuged three times (10,000 rpm, 4°C, 10 min) for removing traces of blood cells. It was then stored at 4°C with sodium azide (0.05%) and also at -70°C in 30 per cent (v/v) of sterile glycerol.

Immunometric assay of E. carotovora var. carotovora protein

Dilutions of the antigen preparation *viz.*, 1:50, 1:100, 1:200, 1:500, 1:1000 and 1:10,000 were made in 0.1 M phosphate buffer, pH 7.0. and various dilutions of the PCA starting from 1:50 to 1:10,000 were also prepared in conjugate buffer. The titre of the polyclonal antibody was tested by indirect (ELISA) explained by Hobbes *et al.* (1987).

Survival of Soft rot Pathogens

The soft rot infected bulbs as well as leaves were collected and it was chopped to approximately 1.0 cm size. These stubble-pieces were mixed in sterilized and unsterilized soil and filled in the pots by the following treatments as given below.

Treatments:

1. Sterilized soil
2. Sterilized soil + leaves
3. Sterilized soil + leaves + bulbs

Table 2. Sources of survival for *Erwinia carotovora* var.*carotovora*

S.No	Sources of Survival	Number of colonies isolated (10^6 CFU/ml)* / months						Mean
		Oct	Nov	Dec	Jan	Feb	Mar	April	
1.	Sterilized soil	105.0^{bc}(2.02)	95.0^{b}(1.98)	82.7^{c}(1.92)	71. 7^{c}(1.86)	40.0^{c}(1.61)	14.0^{c}(1.16)	0.0^{c}(0.30)	58.0 (1.55)
2.	Sterilized soil + Leaves	133.0^{ab}(2.13)	120.0^{ab}(2.08)	100.0^{bc}(2.02)	90.0^{bc}(1.96)	60.0^{b}(1.79)	21.7^{b}(1.34)	1.3^{c}(0.34)	75.3 (1.67)
3.	Sterilized soil + Leaves + Bulbs	167.0^{a}(2.22)	152.0^{a}(2.18)	143.3^{a}(2.16)	125.0^{a}(2.10)	108.0^{a}(2.03)	38.0^{a}(1.59)	20.0^{a}(1.31)	107.6 (1.94)
	Un sterilized soil	81.7^{d}(1.91)	55.0^{c}(1.74)	43.3^{b}(1.64)	31.7^{b}(1.50)	11. 3^{a}(1.07)	1.0^{f}(0.38)	0.0^{e} (0.30)	32.0 (1.22)
	Un sterilized soil + Leaves	98.3^{cd}(1.99)	96.7^{b}(1.99)	85.0^{bc}(1.93)	98.3^{bc}(1.96)	21.7^{d}(1.34)	5.0^{e}(0.74)	0.0^{c}(0.30)	57.9 (1.46)
5.	Un sterilized soil + Leaves + Bulbs	128.3^{b}(2.10)	123.3^{ab}(2.10)	108.3^{b}(2.03)	95.0^{b}(1.98)	35.7^{c}(1.56)	10.7^{d}(1.04)	6.7^{b}(0.85)	72.6 (1.67)
7.	Bulbs alone	135.0^{ab}(2.13)	141.7^{a}(2.15)	0.0^{e}(0.30)	0.0^{d}(0.30)	0.0^{f}(0.30)	0.0^{f}(0.30)	0.0^{e} (0.30)	39.5 (0.83)
	Mean	121.1 (12.07)	111.9 (12.03)	80.3 (1.71)	73.1 (1.66)	39.5 (1.39)	12.9 (0.94)	4.0 (0.53)	63.2 (1.48)

* Values are the mean of three replications

In a column means followed by a common letter are not significantly different from each other at the 5% level by DMRT.

Figures in parentheses are log transformed values

CD (5%) = 0.10

4. Unsterilized soil
5. Unsterilized soil + leaves
6. Unsterilized soil + leaves + bulbs
7. Bulbs alone

To study the survival of the pathogens in the above sources, they were inoculated with 5 per cent of 5x 10^6 CPU ml^{-1} talc based formulation *E. carotovora* var. *carotovora* and samples of about 10g were drawn at monthly intervals and colony loads estimated from each treatment was tabulated (Sundar and Satyavir, 1998).

Management

Biological Method

Isolation of fluorescent pseudomonads

Native onion and tomato rhizosphere strains were isolated from samples obtained from Palladam (PMSI) and orchard of TNAU campus (COHI) respectively. The onion plants were pulled out gently with roots intact and removed. One gram of rhizosphere soil adhering to the root surface was collected and transferred to a 250 ml conical flask containing 100 ml of sterile water. After thorough shaking for 15 min. in shaker, different dilutions were prepared. One ml each of 10^{-5} and 10^{-6} dilutions were pipetted out and poured into the sterile Petri dishes. Later, King's B (KB) medium (King *et al.*, 1954) was poured, rotated and incubated at room temperature (28 ± 2°C) for 24 h. The fluorescent colonies were viewed under uv light at 366 nm and the bacterial strains were characterized (Stonier *et al.*, 1966). Similarly, the same method was followed for isolation of fluorescent pseudomonad strains from tomato rhizosphere soil. The fluorescent pseudomonad strains *viz.*, FP7 and

Pfl were obtained from the culture collection of Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore and used in this experiments.

Screening of bacterial antagonists preparation of talc formulation.

Bacterial antagonists were screened by the method explained by Chakravarthi *et al.* (1972). The talc based formulations of bacterial antagonists were prepared by the method of Vidhyasekaran and Muthamilan (1995).

Physical Methods

The following physical treatments were compared either alone (or) in combination for their efficacy in reducing soft rot disease.

(i) Cold Storage

For low temperature storage studies at 10°C and 20°C an incubator was used.

(ii) Bulb storage in Pyramids

A cardboard pyramid was constructed as per the measurements given by Rama Ranganathan (1995) with the following size of 12cm height, 18.8 cm base length and 17.9 cm side length. The Pyramid aligned on the four cardinals points-north, east, south and west was placed on the floor far away from electrical appliances. Onion bulbs were placed in the pyramid under its apex on the table at a height of one third of the vertical height of the pyramid from the ground for three months. The normal storage in baskets was used for control.

(iii) Sun curing

Sun curing was achieved by exposing to high temperature (noon time) for 4h. The bulbs were stored at ambient conditions in nylon netted bags and observations on storage losses due to decay and Physiological Loss of Weight (PLW) was recorded after 2nd and 3rd months.

Results and Discussion

Source of inoculum

The study on the nature and extent of bacterial contamination in godowns revealed the presence of bacteria in soil particles, leaves, scales, rootlets, godown sweeping and gunny bags, since they serve as a good source of inoculum. *E. carotovora* var. *carotovora* was found to be the predominant bacteria in godown sweeping, gunny bag, soil and root lets. Fungal pathogens like *Aspergillus* sp. and *Fusarium oxysporum* f.sp. *cepae* were also observed in all parts except leaves (Table 1).

De Boer (1979) isolated *E. carotovora* var. *carotovora* from foliage, roots, new tubers and root zone samples collected from potato plants 10 weeks after planting with tuber previously

Table 3. Determination of Antiserum titre value by Immunometric method (Indirect ELISA)

Antigen dilution	Antiserum dilution							
	1:50	1:100	1:200	1:500	1:1000	1:2000	1:5000	1:10000
1:50	0.572	0.564	0.521	0.519	0.476	0.275	0.149	0.149
1:100	0.558	0.524	0.501	0.496	0.461	0.258	0.142	0.142
1:200	0.512	0.504	0.481	0.476	0.450	0.247	0.122	0.128
1:500	0.478	0.402	0.372	0.365	0.355	0.239	0.125	0.122
1:1000	0.385	0.372	0.348	0.341	0.339	0.237	0.116	0.115
1:10000	0.255	0.243	0.235	0.231	0.222	0.221	0.113	0.113

Table 4. Effect of *Pseudomonas fluorescens* strains on the *in vitro* growth *of Erwinia carotovora* var. *carotovora*

Strains of *Pseudomonas*	Inhibition distance (mm)* / days after incubation									Mean
	1	2	3	4	5	6	7	8	9	
FP_7	7.33^{a}	12.00^{a}	14.00^{a}	20.00^{a}	28.00^{a}	34.00^{a}	40.00^{a}	46.67^{a}	49.33^{a}	27.963
PMS1	1.33^{d}	3.33^{e}	3.67^{b}	5.00^{c}	6.67^{d}	12.33^{c}	11.00^{d}	13.00^{d}	15.00^{e}	7.926
COH1	3.00^{c}	4.00^{c}	4.33^{b}	5.00^{c}	8.00^{d}	12.00^{c}	14.00^{a}	17.00^{c}	19.00^{d}	9.593
PF1	7.00^{a}	9.00^{b}	13.00^{a}	14.00^{b}	21.00^{b}	28.67^{a}	37.00^{a}	39.00^{a}	41.00	23.296
Bacillus subtilis	19.00^{ab}	10.33^{f}	18.33^{ab}	22.33^{da}	31.00^{ad}	36.67^{ba}	44.33^{ab}	47.33^{a}	48.32^{a}	28.67
Control	0.00^{d}	0.00^{d}	0.00^{c}	0.00^{d}	0.00^{e}	0.00^{d}	0.00^{e}	0.00^{e}	0.00^{f}	0.00

* Values are the mean of three replications

In a column means followed by a common letter are not significantly different from each other at 5% level by DMRT

CD (5%) - 0.5

inoculated with the causal bacterium *E. carotovora* var. *carotovora*. Tanaka and Saito (1986) reported that *E. carotovora* var. *carotovora* survived in the soil during winter and provided primary inoculum to cause bacterial soft rot of onion. Stanghellini *et al.* (1979) reported that *Erwinia* were consistantly isolated from 23 commercial fields.

An investigation was made to find out the nature and extent of microbial contamination in godowns revealed the occurrence of several postharvest pathogens such as *E. carotovora* var. *carotovora, Pseudomonas allicola* and *Aspergillus niger* which may serve as source of inoculum. The present study indicated the presence of *E. carotovora* var. *carotovora* on the above materials in the godown and further it was confirmed by isolation of *E. carotovora* var. *carotovora* from all the parts except dried outer scale (Table 1). This indicates the need for godown hygiene, sterilization of gunny bags which were repeatedly used. It also warrants the need to avoid heaping of onion in ground, as they serve as the source of inoculum. The need to maintain godown hygiene and sterilization of gunny bags are brought out from the above study.

Source for survival of Erwinia carotovora var. carotovora

Maximum number of colonies were recorded in the first month of inoculation and started reducing thereafter. The mean colony count varied with source of survival and it was ranged from 32 x 10^6 CPU ml^{-1} to 107.4 x 10^6 CPU ml^{-1}. The maximum colony counts were observed in the combination of sterilized soil, leaves and bulb sources while the minimum was recorded in unsterilized soil (32 x 10^6 CPU ml^{-1}) indicating the role of natural antagonists in suppression of the pathogen (Table 2).

Table 5. Effect of seed treatment with bioagents and chemicals on seed germination and growth in onion

S.No.	Biological/ chemical agents	Germination percentage*	Per cent over control	Vigour index*	Per cent increase over control
	A. Biological agents				
1.	*Bacillus subtilis*	40^{b} (39.23)	80.0	7.24	82.73
2.	FP7 *(P. fluorescens)*	83.33^{g} (65.91)	90.39	6.91	81.91
3.	Pf1 *(P. fluorescens)*	64.10^{e} (53.19)	87.52	4.35	71.26
4.	*Trichoderma viride*	52.0^{d} (46.15)	84.61	4.45	71.91
5.	Inoculated control *(Erwinia carotovora var. carotovora)*	1.33^{a} (16.62)	-83.37	0.13	-89.96
	B. Chemical agents				
6.	Bactronol - 100	55.25^{d} (48.01)	85.52	4.19	70.17
7.	Control	8.00^{b} (16.41)		1.25	
	Mean	43.43^{b} (39.36)			

* Values are the mean of three replications.

In a column, means followed by a common letter are not significantly different from each other at 5% level by DMRT Figures in parentheses are arcsine transformed values.
CD (5%) = 0.51

Table 6. Screening of disinfectants against *Erwinia carotovora* var. *carotovora.*

S.No.	Disinfectant	Area of inhibition zone (mm^2)				Mean
		Concentratin (%)				
		0.5	1	1.5	2	
1.	Boric acid	43.74^{b}	112.13^{b}	226.02^{b}	254.59^{c}	159.12
2.	Bleaching powder	3.11^{b}	7.07^{b}	7.07^{c}	19.62^{d}	9.21
3.	Sodium hypochlorite	112.70^{b}	226.04^{b}	345.50^{b}	490.28^{b}	293.63
4.	Formalin	509.48^{a}	493.37^{a}	1808.62^{a}	2731.92^{a}	1385.85
5.	Control	0.0^{b}	0.0^{b}	0.0^{c}	0.0^{d}	0.0
6.	Control	0.0^{c}	0.0^{c}	0.0^{f}	0.0^{f}	0.0
	Mean	133.80	167.72	477.44	699.28	369.56

* Values are the mean of three replications.
In a column means followed by a common letter are not significantly different from each other at 5% level by DMRT.
CD (5%) = 212.75

Table 7. Assessing the suitability of different physical methods of storing onion bulbs against soft rot.

S.No.	Methods	Spoilage (PDI) months after storage			Mean	PLW (%)
		1	2	3		
1.	Pyramid	0.0^{c} (4.05)	0.0^{d} (4.05)	0.0^{d} (4.05)	0.0 (4.05)	2.71(9.10)
2.	10°C incubator	0.0^{c} (4.05)	0.0^{d} (4.05)	0.0^{d} (4.05)	0.0 (4.05)	3.12 (10.08)
3.	20°C incubator	0.0^{c} (4.05)	4.13^{c} (11.67)	3.80^{c} (11.22)	9.26 (8.98)	5.14 (13.07)
4.	Sun cured bulbs	5.90^{b} (13.99)	9.86^{b} (18.27)	13.80^{b} (21.78)	9.86 (18.02)	23.27 (28.83)
5.	Control	38.43^{a} (38.31)	17.03^{a} (24.34)	24.83^{a} (29.85)	26.77 (30.84)	66.63 (54.74)
	Mean	8.87 (12.89)	6.21 (12.48)	8.49 (14.19)	7.85 (13.19)	20.177 (23.17)

Average of three replication.
In a column means followed by a common letter are not significantly different from each other at 5% level by DMRT.
CD (5%) = 1.88
Figures in parentheses are arcsine transformed values.

Information on the survival and source of inoculum are the prerequisites for developing effective management strategy. Survival period of *E. carotovora* var. *carotovora* was tested in different sources. Sterilized soil + leaves + bulbs has high number of colonies at 6 months after inoculation whereas unsterilized soil + leaves + bulbs has half of the bacterial colony from previous treatments at the same period. The present study showed that the soft rot bacterium survived in the sterilized medium till season was over. While in unsterilized soil half of the inoculum load was retained upto a period of six months making the inoculum available to the subsequent crop thus serving as a source of primary inoculum. The same findings supported by De Borer *et al.* (1979), Tanak and Saito (1986) and Stanghnelli *et al.* (1979) in carrot field.

Detection of bacteria from seed

Antibodies raised against *E. carotovora* var. *carotovora* were diluted upto 1:10000. They were tested with same similar dilution of antigen. Antiserum could detect its antigen upto 1:2000 dilution, when the dilution of antigen was 1:500 and 1:1000. However, when the antigen was diluted to 1: 200 such detection was possible only when the antiserum was not beyond 1: 1000 (Table 3).

Previously Lin *et al,* (1987) raised polyclonal antibodies against *E. amylovora* in apple. Keil and van der Zwet (1972) developed polyclonal antiserum against *Pseudomonas phaseolicola* for the detection of the pathogen from seed lot of beans.

Effect of Biocontrol agents against E. carotovora var. carotovora under in vitro *condition*

Among the biocontrol agents tested, *Pseudomonas* strain FP7 and *Bacillus subtilis* were found to be promising in reducing the growth of soft rot bacteria under *in vitro* conditions.

The results obtained are similar to the reports of Schistler *et al.* (1995) who stated that *Pseudomonas fluorescens* was able to check *E. carotovora* var. *carotovora* under *in vitro* conditions. Sharjg and Lyoa (1998) reported that *Bacillus subtilis* showed antimicrobial activity

against *E. carotovora* var. *carotovora* and *Bacillus subtilis* was resistant to the action of nucleases, proteases and lipases of the same pathogen.

Seed treatment with biocontrol agents and chemicals

Among the biocontrol agents and chemicals tested *Pseudomonas fluorescencs* FP7 showed reduced incidence and increased growth of seedling followed by Pfl strains of *Pseudomonas fluorescens* (Table 6). Many workers believed that the plant growth promoting activity of fluorescent pseudomonads may be due to extra cellular siderophores formation (Kloepper *et al* 1980), antibiotic and toxic action (Weller, 1988) or may be due to the production of growth promoting hormones by PGPR (Barea *et al.* 1976; Dubeikovsky *et al.* 1993). Mew and Rosales (1986) observed an increase in plant height and tiller number in rice due to bacterial seed treatment. They also stated that the plants from treated seeds grew greener than the plants from untreated seeds.

Similar findings were also reported by Weller and Cook (1983) and Capper and Higgins (1993) while working with take all disease of wheat.

In the present investigation, the strains *viz.*, FP7 and Pfl increased the plant height and tiller number under green house conditions. Though the strain FP7 increased the growth when compared to control, it was not on par with the other strains. Significant differences in plant height existed between treatments.

Storage methods

Effect of physical methods of storing onion on soft rot incidence

Of the four physical methods of storage evaluated for their efficacy in controlling storage rot, the pyramid method of storage and storing the bulbs in an incubator at 10°C was superior compared to others as this method have recorded no soft rot incidence.

When the bulbs were stored after sun curing, highest percentage of rotting (23.27%) was observed. After 90 days of storage, bulbs stored in an incubator at 20°C recorded 5.14 per cent of Physiological Loss of Weight (PLW) when compared to 2.71 per cent PLW in pyramid storage (Table. 6)

Tandon and Mishra (1969) highlighted the importance of storage of papaya fruits at 10°C for controlling *Rhizopus* rot and *Colletotrichum* rot respectively. Storage of fruits in pyramids was attempted by Rama Ranganathan (1995) using tomato and grapes, he reported a significant increase in shelf life, as observed in the present study. Pramod and Palaniswami (1999) stated that storage of fruits in pyramids also increased shelf life.

Screening of disinfectants against E. carotovora var. carotovora

Formalin showed maximum inhibition zone of 1385.85 mm^2. Other disinfectants *viz.* boric acid, bleaching powder and sodium hypochlorite though less effective, were superior to control (Table 7.)

Repeated storage of diseased bulbs in the same godown continuously results in contamination of the godowns and hence has to be disinfected properly. In the present study formalin (2 per cent) arrested the growth of *E. carotovora* var. *carotovora.*

Shashirekha and Narasimham (1990) stated that the disinfectants were found to exert *in vivo* antimicrobial activity against *E. carotovora* var. *carotovora* and *Fusarium oxysporum.*

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