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Ecofriendly management of post-harvest bacterial soft rot of potato caused by *Pectobacterium carotovorum* using different botanicals

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Abstract
Bacterial soft rot caused by *Erwinia carotovora* is a major bacterial disease infecting almost all vegetable species having fleshy storage organs including potato. Management of post-harvest bacterial soft rot by chemicals has not proven practical and use of antibiotics can lead to severe health issues. During present study attempts were made to use aqueous plant extracts for the management of this disease. Aqueous extracts of 14 plant species, most of them belonging to the temperate ecology, were evaluated against the pathogen *in vitro*. Among them *Syzygium aromaticum* and *Salix alba*proved highly effective against the pathogen. *Azadirachta indica*, *Urtica dioica*, *Xanthium* sp., *Cupressus torulosa* and *Morus alba* also inhibited the growth of pathogen *in vitro*. *Pinus* sp, *Allium sativum*, *Artemisia annua*, *Juglans regia* showed moderate inhibition while aqueous plant extracts of *Plantago major* and *Mentha arvensis* were least effective. *Syzygium aromaticum*, *Salix alba* and *Azadirachta indica* which proved effective against the growth of test pathogen in-vitro, were also evaluated on potato tubers inoculated with the pathogen at different times i.e 12 hours prior to, simultaneously and 12 hours after the inoculation of the pathogen. Among these extracts, *Syzygium aromaticum* showed highest control of disease followed by *Salix alba* and *Azadirachta indica* in reducing the severity of disease recorded up to 6 days of storage. Best results were obtained with treatments given 12 hours prior to and at the time of inoculation of the pathogen.

Keywords: Aqueous plant extracts, *Erwinia carotovora*, potato, post-harvest soft rot

Introduction
Bacterial soft rot of potato has become a very important disease world-wide due to the losses it causes during the various stages of crop development and in storage (Reeves *et al*., 1999; Bdilya and Bashir, 2006) [17, 4]. *Pectobacterium* (*Erwinia*) is known to cause soft rot, blackleg and wilting diseases that cause huge economic losses (Baghaee-Ravari *et al*., 2011) [2]. Although soft rot in potato and in different vegetables is caused by various species of bacteria, but among them, *Erwinia carotovora* subsp. *carotovora* is considered to be one of the major soft rot causing bacteriumwhich is commonly associated with soft rot tubers of potatoes (Perombelon and Kelman, 1987; Larka, 2004; Rahmanifar *et al*., 2012) [13, 9, 16] and many other vegetables having fleshy storage organs.
Since the disease is destructive especially in transit and in storage,wide range of chemicals and antibiotics are in use against it with somewhat satisfactory results. However, prolonged use of antibiotics led to the bacterial adaptation, resulting in the development of multidrug resistance in bacteria leading to several human health complications. This has significantly limited the use of antibiotics, warranting alternative strategies to combat rot causing microbes. Moreover, use of antibiotics on vegetables during storage exposes the vegetable consuming populations to low doses of antibiotics thereby, making human pathogenic bacteria resistant to them. The emergence of antibiotic resistance in pathogenic bacteria has led to renewed interest in exploring the potential of plant derived antimicrobials or antagonistic bio-agents as an alternative strategy to combat these microbes which are safe, effective, economical, without any side effects and are readily available in nature. As target sites of action is highly diverse in case of these plant extracts, it is difficult for microorganisms to create resistance against these (Thompson *et al*., 2013; Rahman *et al*., 2011) [22, 15]. Since antimicrobial potential of most of the temperate plants remained untapped and none of the previous studies evaluating temperate plants of Kashmir against Bacterial soft rot of Potato or any other host has been conducted, the present study was taken up to find any possible botanical alternative to combat this disease.

Material and Methods
Collection of plant specimens: Extracts of fourteen plant species were used during the present studies. Out of these eleven species were native to Kashmir valley, while parts of three species

used were brought from other regions. Plant species were identified by Division of Agronomy, Faculty of Agriculture, Sher-e-kashmir University of Agricultural Sciences and Technology. List of plants and their parts used are as under.

Plants species	Parts used
Syzygium aromaticum	Floral buds
Salix alba	Leaves
Azadirachta indica	Seed kernel
Eucalyptus sp.	Leaves
Urtica dioica	Leaves
Xanthium sp.	Leaves
Juglans regia	Fruit green hull
Cupressus torulosa	Leaves
Pinus sp.	Needles
Artemisia absinthium	Leaves
Morus alba	Leaves
Plantago major	Leaves
Mentha arvensis	Leaves
Allium sativum	Cloves

Preparation of aqueous plant extracts: Collected plant material was shade dried and grinded into fine powder in an electrical blender. 10% extract was prepared by mixing 10g of powder in 100ml water by boiling at 100 ºC on water bath for 20 minutes. Resultant suspensions obtained were filtered through Whatmann filter paper 1 and the concentrated filtrate material stored in glass bottles at 4 ºC in refrigerator for further studies.

Isolation of the causal pathogen: Diseased vegetables and potato tubers showing typical soft rot symptoms were collected from the local markets from Kashmir valley. Collected samples were surface sterilized with 0.1% sodium hypochlorite solution and the infected tissue was macerated in sterile water to make a bacterial suspension. A drop of resultant suspension was spread on Crystal violet pectate, a semi selective medium (CVP). The type of colonies which upon flooding with 1% hexadecyl trimethyl ammonium bromide (precipitant solution) formed halo zones around them on Crystal violet pectate medium (CVP) were selected for subculturing on nutrient agar and were tested for pathogenicity.

Pathogenicity test

Tuber hole inoculation method. The tubers were surface sterilized in 0.1% sodium hypochlorite solution for three minutes followed by five serial washings with sterile tap water. Tubers were allowed to dry at room temperature. The tubers were wounded by punching 3 holes about 5 mm deep using 2 mm thick cork borer. Next, the tubers were artificially inoculated by adding the cell suspension (1×10^8 cfu/ml) of test bacterium in the holes. The inoculated tubers were covered by jars along with moist filter papers in order to maintain humid conditions and these were kept for incubation at 30ºC (Bdilya and Bashir, 2006) [4]. The pathogen showing typical soft rot symptoms was reisolated from the tubers and was further characterized by biochemical and other morphological tests to ascertain identity. Further help was also taken from ITCC, New Delhi for precise identification of the causal pathogen.

***In-vitro* screening of Antibacterial activity of plant extracts**: Nutrient agar medium was prepared. About 15ml of nutrient agar medium was poured in sterile 10 cm Petri plates and was allowed to solidify and then 24hour old bacterial culture was taken and mixed with water to make a bacterial suspension, from which 0.5ml of the bacterial inoculum containing 1×10^8cfu/ml was flooded on the surface of nutrient agar plates and was spread all over by glass spreader. Subsequently, strile filter paper discs (6mm diameter) impregnated with the test extracts by dipping in plant extract were placed on the surface of the agar at equidistant points using sterile forceps. Plates were incubated at 30 ºC for 24 hours. Antibacterial activity was evaluated by measuring the diameter of the zone of inhibition to the nearest millimetre using ruler. Three discs (comprising of three replications) were kept in each petri plate. Discs saturated with sterile water and antibiotic (Streptomycin @ 150ppm) were kept as negative and positive controls respectively (Las llagas *et al.*, 2014) [10].

Screening of effective Plant extracts against the disease on stored potato tubers:

Plant extracts which proved best *invitro* were used for the treatment on stored potato tubers and applied at different times *viz.* 12 hours prior to the inoculation of the pathogen, simultaneously with the inoculation of the pathogen and 12 hours after the inoculation of the pathogen. Fresh potato tubers were surface sterilized by dipping in 0.1% solution of sodium hypochlorite followed by serial washings with sterile water and then dried under the hood of laminar air flow. One set of potato tubers was given 30 pinpricks and dipped in uniform suspensions of different plant extracts for 10 minutes and 12 hours afterwards inoculated with the pathogen by swabbing the bacterial suspension on them. In the second set of tubers, after giving the pinpricks and inoculating them with pathogen, application of plant extracts was done simultaneously. In third case, the pinpricked tubers were first inoculated by the pathogen by swabbing bacterial suspension on them and 12 hours afterwards they were treated with plant extracts for 10 minutes.

One set of potato tubers which were inoculated with only pathogen (no treatment) served as inoculated control. Other set of tubers inoculated and treated with antibiotic (streptomycin@150ppm) were kept as positive control. Five potato tubers constituted 1 replication and total of 5 replications were maintained in each treatment. The tubers were kept in sterile air tight plastic bags and were stored at 30±1 ºC. Observations on soft rot incidence and severity were recorded on 2nd,4th and 6th day of incubation.

$$\text{Incidence of soft rot disease} = \frac{\text{Number of tubers infected}}{\text{Total number of tubers assessed}} \times 100$$

Tuber rot severity

Severity of the disease was calculated using 0-5 scale (Bdliya and Langerfeld, 2005)

0. No symptoms of rot
1. 1-15% tuber rot
2. 16-30% tuber rot
3. 31-45% tuber rot
4. 46-60% tuber rot
5. ≥61% tuber rot

The severity was calculated using formula:

$$\text{Tuber rot severity} = \frac{\sum nv \times 100}{N \times G}$$

Where,

$\sum$ = Summation

V = Disease score

N = Number of tubers showing a particular score.
N = Number of tubers examined.
G = Highest score.

Data analysis

The collected data was subjected to the analysis of variance using CRD (Complete Randomised Design) and transformed values of the data compared using critical difference (CD) at 5% level of significance using Statistical Package for Agricultural Research workers (OPSTAT).

Results and Discussion

The causal pathogen: The bacterium isolated from potato tubers was a short rod measuring 0.7-1.0µm in width,1-2.5µm in length, Gram negative, facultative anaerobic, produced acid from D lactose, trehalose and maltose, did not hydrolyze the starch, reduced nitrates, liquefied gelatin, degraded pectin, produced H_2S from cysteine, was catalase positive, oxidase negative, urease negative, not sensitive to erythromycin and showed positive growth at 37ºC. Based on Morpho-cultural, biochemical and pathogenic characters, the pathogen was identified as *Erwinia carotovora.* It was also identified as *Erwinia carotovora* by ITCC (Indian Type Culture Collection, IARI, New Delhi as per their communication dated: 04/05/2017 bearing receipt No: DD/RF/2016-17/108.

Fig 1: Pathogenicity on potato tuber a) Soft rot symptoms after 15 hours after inoculation at three points on tuber. b) Symptoms after 3 days

Fig 2: Morpho-cultural characters of the causal pathogen a) Colony color appearing pale white on nutrient agar. b) Micrograph showing bacterium is Gram negative and short rod shaped

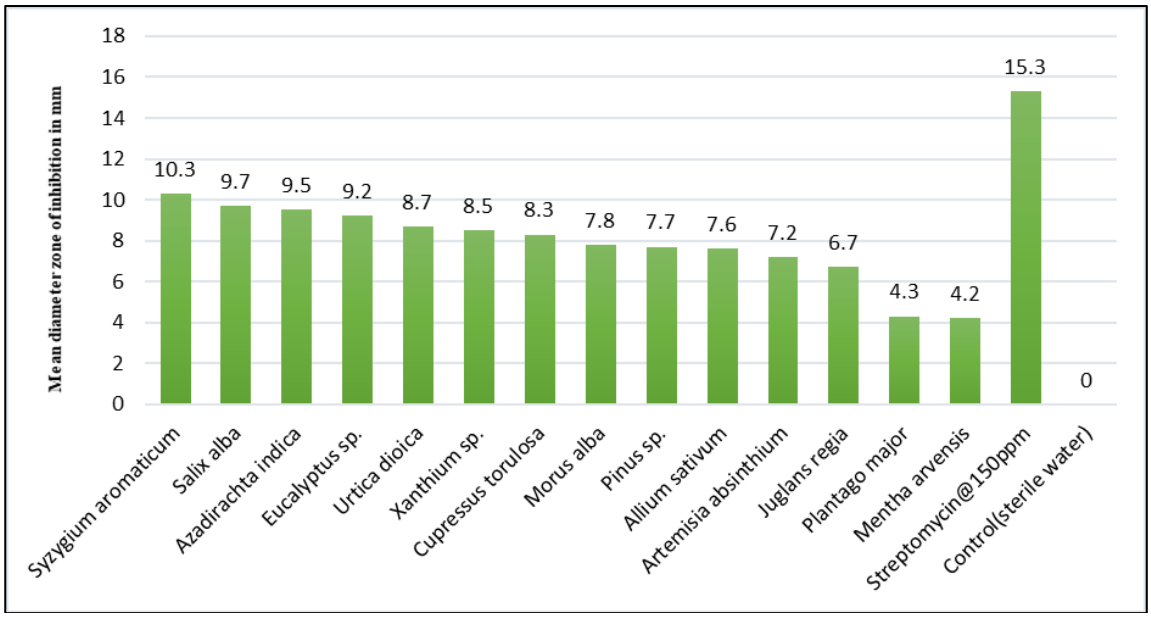


Fig 3: Effect of aqueous plant extracts against the growth of *Erwinia carotovorain-vitro*

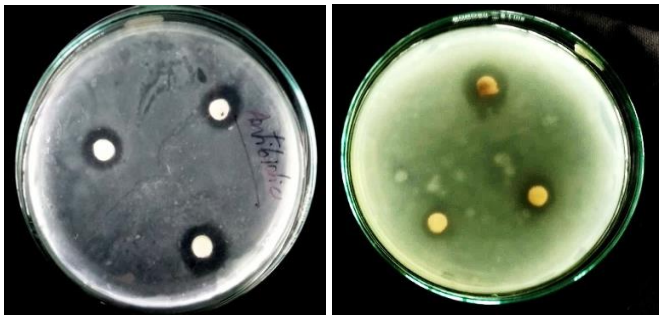


Fig 4: Effect of 10% aqueous plant extracts on the growth of *Erwinia carotovora* a) Streptomycin@150ppm b) *Syzygium aromaticum*

Laboratory evaluation of aqueous plant extracts against the growth of *Erwinia carotovora* in-vitro.

Among 14 aqueous plant extracts evaluated against the growth of *Erwinia carotovora*, highest control was exhibited by *Syzygium aromaticum* (cloves) with highest mean diameter zone of inhibition of 10.3mm (including the disc diameter 6mm), followed by *Salix alba* (leaves) with a zone of 9.7mm. Other plant extracts which followed in decreasing order of efficacy were *Azadirachta indica* (seed kernel), *Eucalyptus* sp.(leaves), *Urtica dioica* (leaves), *Xanthium*sp. (leaves), *Cupressus torulosa* (leaves) and *Morus alba* (leaves). Whereas, extracts of *Pinus* sp (needles), *Allium sativum* (cloves), *Artemisia absinthium* (leaves) and *Juglans regia* (fruit green hull) showed moderate efficacy against the test

bacterium. Least efficacy was exhibited by *Plantago major*(leaves) and *Mentha arvensis* (leaves). Standard check (streptomycin @ 150ppm) gave mean diameter zone of inhibition of 15.3mm (Fig: 3).

We have not come across any previous report concerning the efficacy of aqueous plant extracts of *Syzygium aromaticum, salix alba, Urtica dioica* (leaves), *Xanthium* sp. (leaves), *Cupressus torulosa* (leaves) *Morus alba* (leaves), *Pinus* sp. (needles), *Artemisia absinthium* and *Juglans regia* against *Erwinia carotovora* the causal pathogen of soft rot of vegetables. Growth inhibition of *Erwinia carotovora* by aqueous extracts of *Allium sativum* has been previously reported by Akbar *et al.* 2014 [1]. In-vitro growth inhibition of *Erwinia carotovora*by aqueous extracts of *Azadirachta indica*and *Eucalyptus sp*. was also reported (Akbar *et al*., 2014; Opara and Agugo, 2014; Simeon and Abubakar, 2014) [1, 11, 20]. Present studyrevealed that aqueous plant extracts of *Syzygium aromaticum* and *Salix alba* are having higher efficacy against *Erwinia carotovora* than *Azadirachta indica*. *Urtica dioica* (Leaves), *Xanthium* sp. (Leaves) and *Cupressus torulosa* (Leaves) *Morus alba* (Leaves), *Pinus* sp (Needles) also gave a satisfactory inhibition against the test pathogen, while as moderate to low inhibition was obtained *by Allium sativum, Artemisia absinthium* (Leaves), *Juglans regia* (Fruit shell), *Plantago major* (Leaves) and *Mentha arvensis* (Leaves). A low inhibition by aqueous plant extract by *Mentha arvensis* was also reported by Akbar *et al*. 2014 [1]. Thus out of aqueous extracts of14 plants tested, 11 plant species mostly of temperate ecology are being probably reported for the first time against *Erwinia carotovora*, Although amongst them most aqueous extracts have been proven effective against other bacterial species pathogenic to animals. Aqueous infusion and essential oil of clove (*Syzygium aromaticum*) was found to exhibit antibacterial activity against several Gram negative bacteria *in-vitro* (Saeed and Tariq, 2008) [18]. Sofia *et al*. (2007) [21] found complete bactericidal effect against all the food borne pathogens tested viz., *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus* by aqueous extract of clove (*Syzygium aromaticum*). Hydrophilic leaf extracts of *Juglans regia* and leaf extract of *Salix alba* was found effective against *E. Coli, Staphylococcus aureus, Listeria monocytogenes, Bacillus cereus* and *Salmonella enteritis* (Pop *et al*., 2013; Shah *et al*.,2013) [14, 19]. Aqueous extract of leaves, barks, fruits and green husks of *J. regia* revealed broad spectrum antibacterial activity against *Pseudomonas aeruginosa* and *E. coli* (Deshpande *et al*., 2011) [6]. Growth inhibition by aqueous leaf extracts of *Morus alba, Xanthium* sp. aerial parts of *Urtica dioica, Artemisia* sp have been reported against wide range of bacteria *viz. E.coli, Bacillus cereus, Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus* (Jha and Srivatsava, 2013; Hassan *et al*., 2014; Das *et al*., 2012; Oriakpono *et al.*, 2010) [8, 7, 5, 12]

Effect of aqueous plant extracts on incidence and severity of soft rot disease caused by *Erwinia carotovora* on stored potato tubers:

Among 14 plants tested in-vitro against the growth of *Erwinia carotovora*, three plant extracts showing highest efficacy with respect to growth inhibition are selected for this experiment. Best results were obtained when treatments were given 12 hours prior to inoculation or simultaneously with the inoculation of the pathogen than when treatments were given 12 hours after the inoculation of the pathogen.

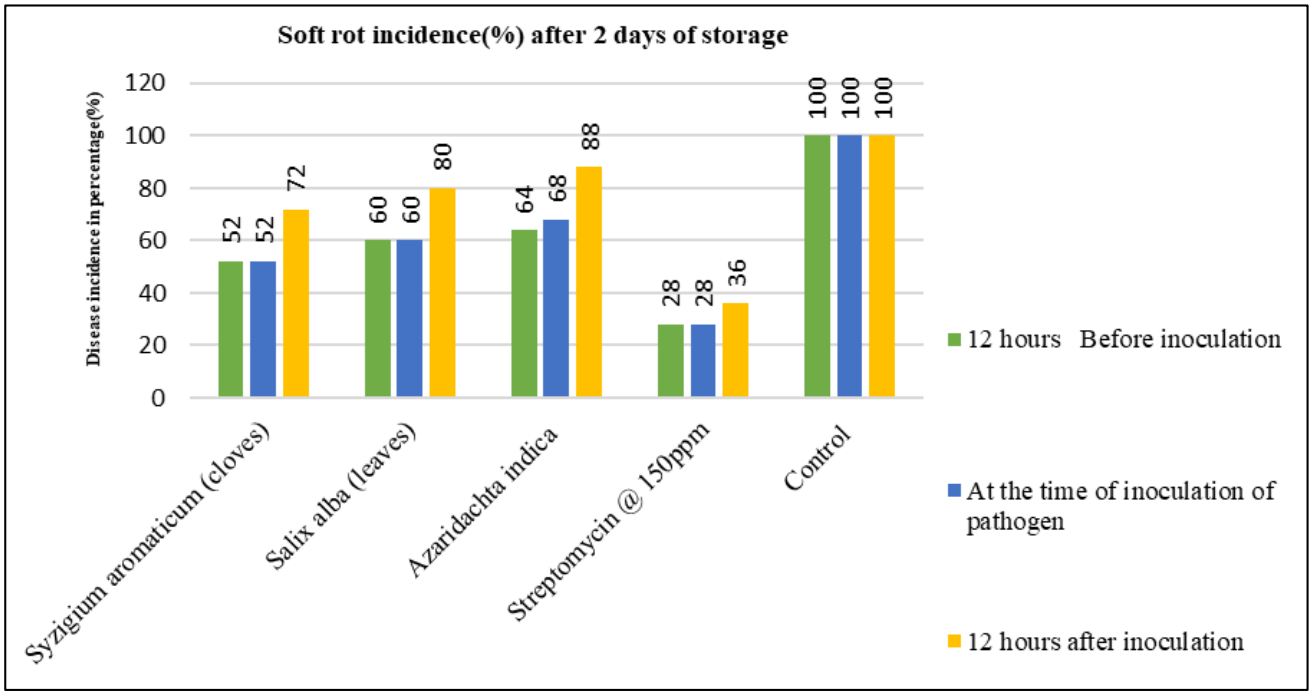
Results obtained after 2 days of storage exhibited that *Syzygium aromaticum* gave least soft rot incidence of52% and severities of 8% and 8.8% when applied 12 hours prior to and simultaneously with the inoculation of the pathogen respectively, followed by *Salix alba* with 60% disease incidence and severity of 9.6% and 11.2%, respectively. *Azadirachta indica* was recorded next to the above plants in efficacy with incidence of 64% and 68% and severities of 12.8% and 13.6% when applied 12 hours prior to and simultaneously with the inoculation of the pathogen. Disease Incidence and severity recorded in case of these extracts was significantly less than that of the inoculated control (pathogen only and no treatment), which showed 100% soft rot incidence and 84.8% disease severity. Results are in Fig-6 (a, b).

After 4 days of storage, there was a rapid progression of disease in case of inoculated control. Disease progression was less in case of treatments. Least disease incidence of80% and 84% and severity of 15.2% and 16.4%, respectively, were recorded in case of treatment with *Syzygium aromaticum*, when applied 12 hours prior to and simultaneously with the inoculation of the pathogen, followed by *Salix alba* with incidence of 88% in both cases and severity of 19.2 and 21.6% when applied 12 hours prior to pathogen inoculation or simultaneously with it, respectively, whereas in *Azadirachta indica* incidences of 92% and 96% and severities of22.4% and 23.6% were recorded when applied 12 hours prior to and simultaneously respectively. Whereas in the inoculated control disease severity of 100% was recorded Fig-7(a, b).

Results obtained after 6 days of storage almost revealed the same trend. Positive check streptomycin showed incidence of 76% when applied 12 hours prior to or simultaneously with the inoculation of the pathogen even after 6 days of storage. There was no hike in the severity of the disease in case of tubers treated with plant extracts. Treatments with plant extracts protected the tubers from speedy spoilage thereby significantly reducing the severity of disease even after 6 days of storage. *Syzygium aromaticum* stands best in preventing the severity of disease by exhibiting least severity of 25.6%, when applied 12 hours before inoculation and 26.4%, when applied simultaneously with the inoculation of the pathogen, followed by *Salix alba* with severities of 29.6% and 31.2% respectively, when applied 12 hours prior to and simultaneously with the inoculation of the pathogen. Wherea, *Azaridachta indica* was next to the above plant extracts in efficacy exhibiting severities of 33.6% and 34.4%, when applied prior to and simultaneously with the pathogen respectively. All these extracts were highly significant and superior to the inoculated control (only pathogen and no treatment Fig-8 (a, b).

A thorough search of literature could not reveal any reports regarding control of soft rotca used by *Erwinia carotovora* in particular by aqueous extracts of Cloves (*Syzygium aromaticum*) and *Salix alba*. But outstanding efficacy of neem seed kernel (*Azadirachta indica*) against post-harvest bacterial soft rot on stored potato tubers caused by *Erwinia carotovora* has already been *reported* by Bdilya and Bashir (2006) [4] which was in consonance with our results. However, during the course of present study, aqueous extracts of *Syzygium aromaticum* and *Salix alba*(leaves) were found more effective than *Azadirachta indica* in decreasing the incidence and severity of post-harvest soft rot caused by *Erwinia carotovora* up to one week of storage. Hence this study proved the potential of plant extracts of *Syzygium aromaticum* and *Salix alba* for their antimicrobial activity and possibility of developing their use against post-harvest soft rot of vegetables in further which are eco-friendly in nature.

Fig 5: Effect of aqueous plant extracts on tuber soft rot on potato caused by *Erwinia carotovora* after 2 days of storage a) Treatment with *Salix alba* b) Treatment with *Azadirachta indica* c) Treatment with *Syzygium aromaticum*



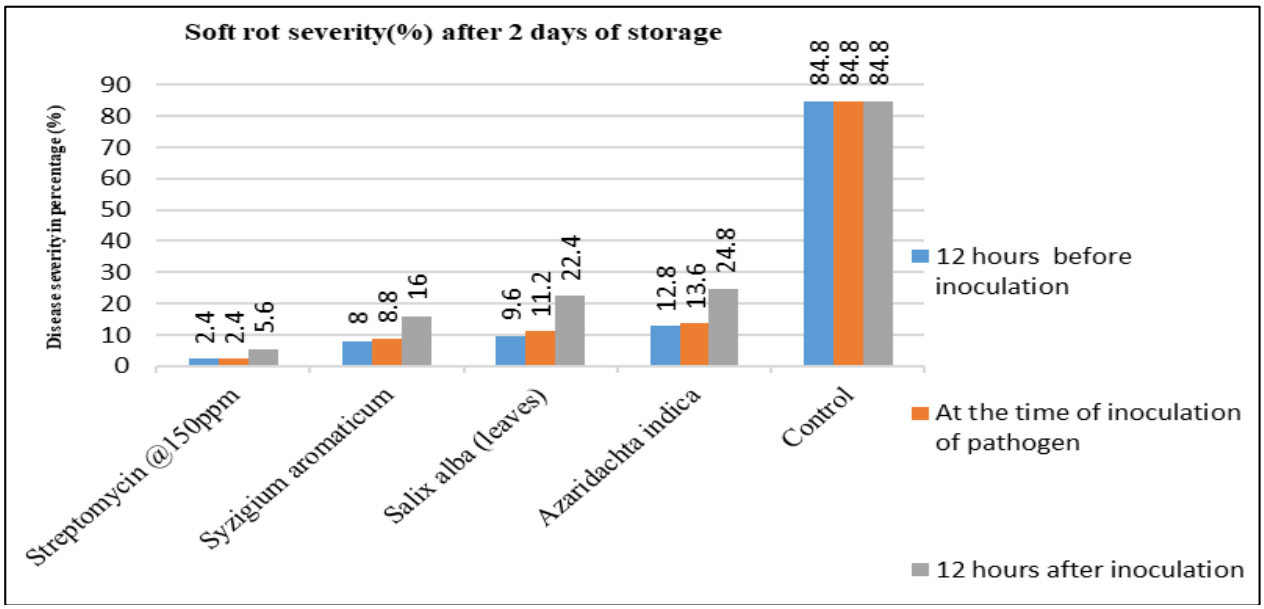
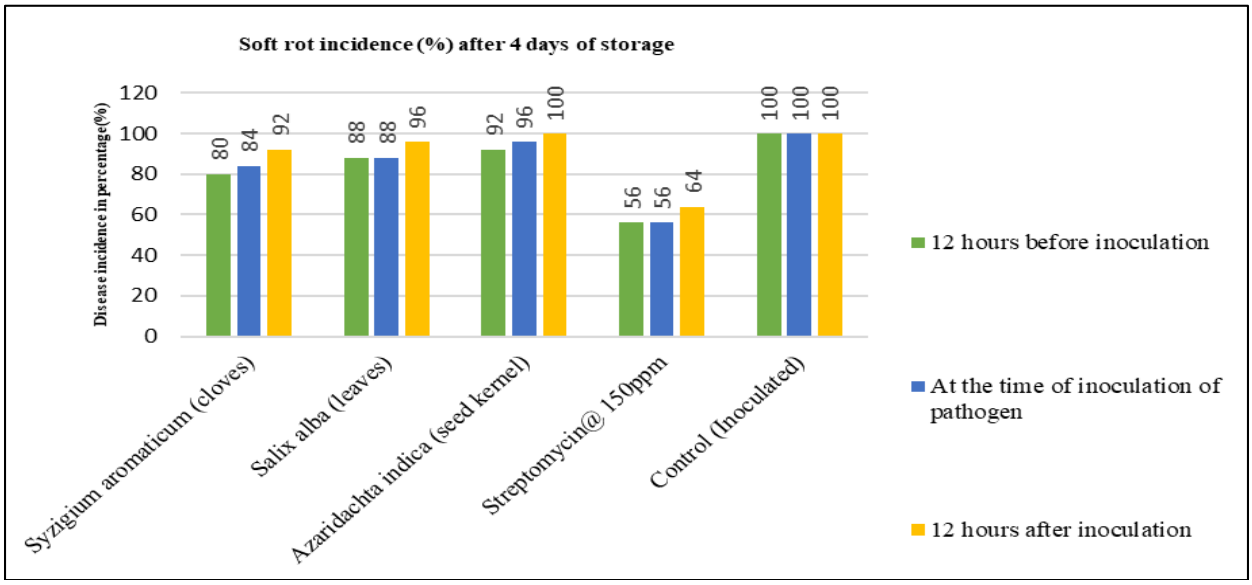


Fig 6: Effect of aqueous plant extracts on post-harvest tuber soft rot after 2 days of storage a: Disease incidence after 2 days of storage on stored potato tubers. b: Disease severity after 2 days of storage on stored potato tubers.



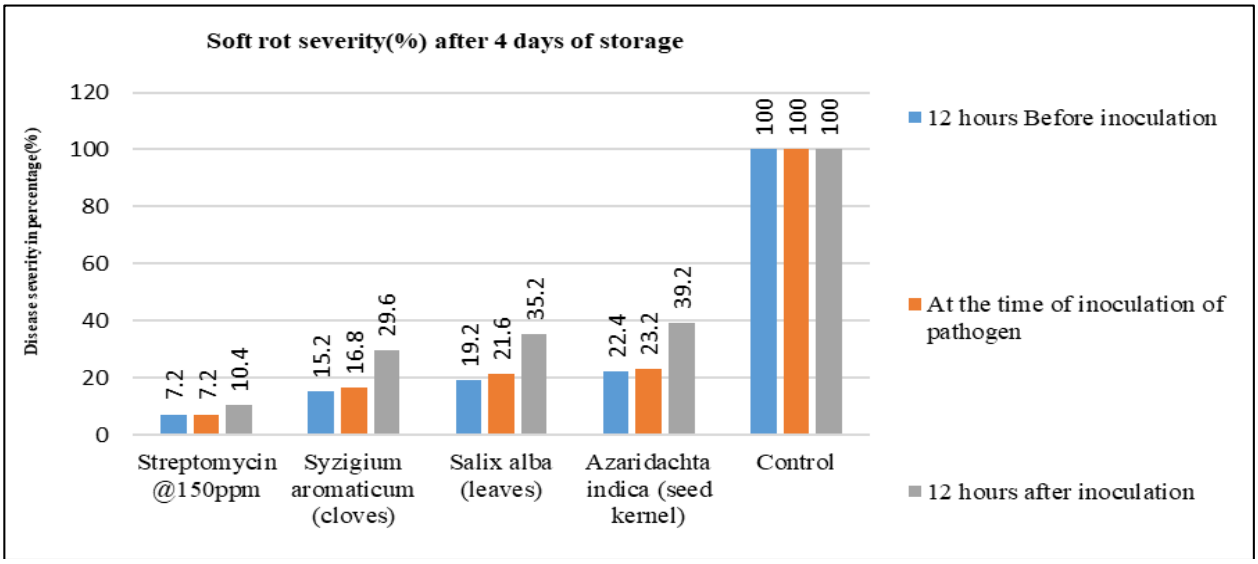
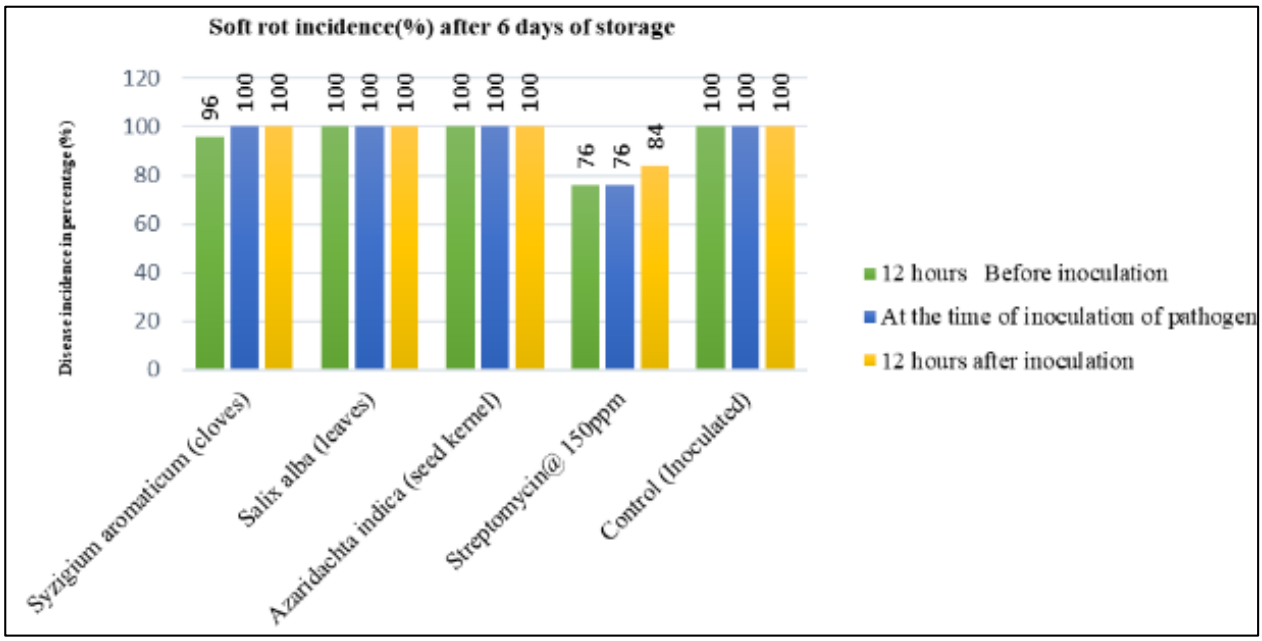


Fig 7: Effect of aqueous plant extracts on post-harvest tuber soft rot after 4 days of storage a: Disease incidence after 4 days of storage on stored potato tubers. b: Disease severity after 4 days of storage on stored potato tubers.



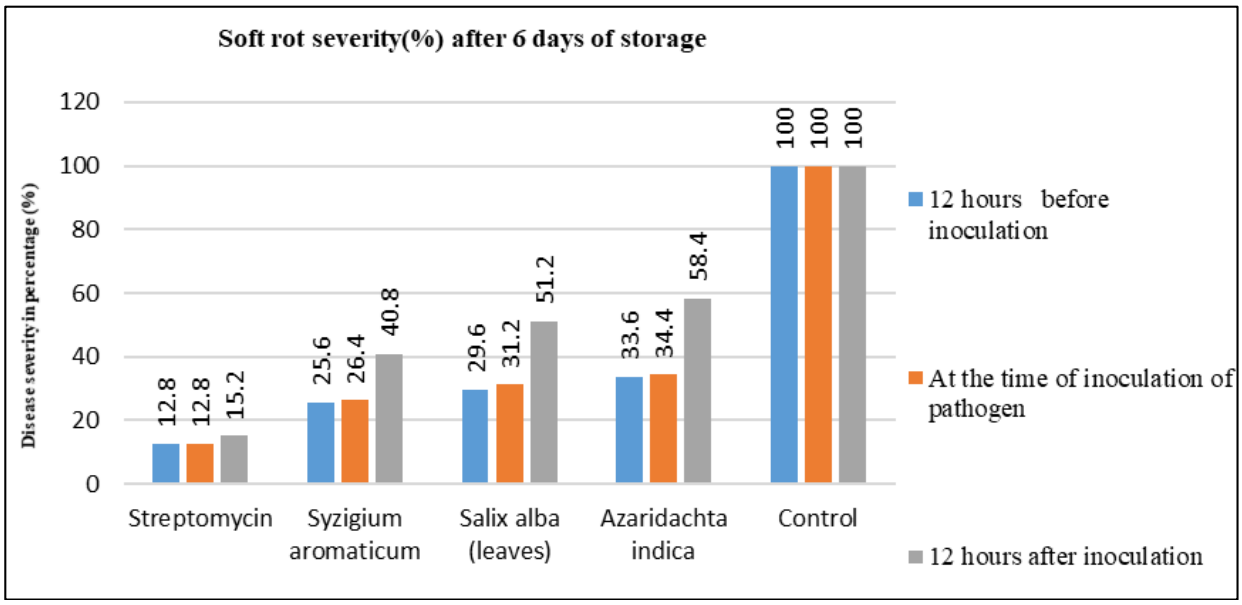


Fig 8: Effect of aqueous plant extracts on post-harvest tuber soft rot after 6 days of storage a: Disease incidence after 6 days of storage on stored potato tubers. b: Disease severity after 6 days of storage on stored potato tubers.

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