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Studies on pre-harvest fruit drop of Kinnow with special reference to disease management through integrated approaches under Punjab Region

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Abstract
Kinnow is an important citrus crop grown in India. Pre-harvest fruit drop caused by fungal pathogens is a major problem with Kinnow mandarin in all fruit growing regions of the world, results in huge economic losses to Kinnow fruit growers**.** In order to measure the pathological fruit drop a survey was conducted in Kinnow growing different regions of Punjab *viz*; Bathinda, Sri Muktsar Sahib and Fazilka. Our results indicated that, Fruit drop was significantly higher during the second fortnight of September and first fortnight of October in Kinnow growing regions of Bathinda, Sri Muktsar Sahib and Fazilka. Three fungi *viz*; *Colletotrichum gloeosporioides*, *Diplodia natalensis* and *Alternaria citri* are considered to be largely associated with the fruit drop disease. All botanicals, BCAs and chemicals inhibited the growth (colony diameter) of both pathogens over untreated PDA plates, but the maximum inhibition was exhibited by *B. subtilis* followed by garlic. It is evident from our studies that BCAs and botanicals have the potential to control Preharvest diseases without causing any injury or harmful effects on Kinnow mandarin; these can be recommended as a safe method for extending its storage life while maintaining fruit quality at the same time.

Keywords: Botanical control, fruit drop, Preharvest pathogens, integrated disease management

Introduction
Citrus cultivars have a major role in the economy of world and ranks among top three fruits of the world with respect to area and production. Among the citrus growing countries, India holds sixth position. Citrus holds third position in fruit industry of India and occupies an area of 9, 55, 200 ha with annual production of 94, 52, 100 MT. At the moment, citrus is being grown in Punjab over 52,836 hectares with annual production of 10,49,977 tonnes. Kinnow mandarin occupies an area of 49,356 hectare with annual production of 10,21,719 tonnes. (Rattanpal *et al.*, 2017) [31].

Cultivation is mainly concentrated in districts of Fazilka, Ferozpur, Faridkot, Bathinda, Muktsar and Hoshiarpur. Among the various pathological constraints, pre-harvest fruit drop is a wide spread disease and every year results in huge economic losses to Kinnow growers. Disease appears brown discoloration near stem-end of fruits due to *Colletotrichum gloeosporioides* was predominantly associated with stem-end rot in the form of firm to semi pliable small brown areas around the stem-end As the area enlarges a soft and pliable rot develops on the fruits. Another fungus which is responsible for pathological fruit drop is *Diplodia natalensis*, *Alternaria citri* is the casual organism of brown spot of citrus. Different pathotypes are associated with the diseases that are characterized on the bases of host specificity. (Sadowsky *et al.*, 2002) [33]. According to Thind and Kumar (2008) [36], the physiological (40-63%) and pathological (5-25%) drop in Kinnow is matter of great concern as the pre-harvest drop results in direct economic losses to the growers. The pathogens associated with the disease have not been studied systematically. Restrictions on fungicide use and widespread emergence of pathogen resistance has increased global demand for more sustainable production systems and driven research towards alternative disease control strategies. The term biocontrol applies to the use of plant extracts and microbial antagonists to suppress diseases. Biological control of plant diseases including fungal pathogen has been considered a viable alternative method to chemical control. Biocontrol agents may provide a seemingly environmental friendly alternative to potent and toxic fungicides, which cannot be broken down in the environment (Abdalla *et al*. 2014).

Some eco friendly plant extracts have been shown to have great potential as an alternative to synthetic fungicides (Janisiewicz *et al.*, 2002) [16]. (Kupper *et al*., 2011) [21] observed that the bacteria *Bacillus subtilis* and fungus *Trichoderma* spp. isolated from citrus plants and citrus oils were shown to inhibit mycelial growth of *G. citricarpa* as an antagonist. In citrus, several bacteria such as *Bacillus* spp. have been reported to reduce postharvest decay Obagwu and Korsten (2003) [29]. Lopes *et al*. (2015) [23] assessed the efficacy of different antagonistic microorganisms in controlling Colletotrichum acutatum with applications on preharvest citrus fruit and found promising results. Plant extracts, essential oils, gums, resins etc, *have* been shown to exert biological activity against plant fungal pathogens *in vitro* and *in vivo* and can be used as bio-fungicidal products (Fawzi *et al*, 2009; Jalili *et al*, 2010; Romanazzi *et al*, 2012) [8, 15, 32]. Many reports approve the efficacy of natural products of plants in controlling fungal growth and mycotoxin production, eg,. Cinnamon, clove, oregano, palmarosa and lemongrass oils (Marin *et al*, 2004) [25], common thyme, cinnamon leaf and aniseed oils (Ćosić *et al*, 2010) [7], sweet basil, neem, eucalyptus, datura, garlic and oleander extracts (Nashwa and Abo-Elyousr, 2012) [27]. The objective of the present study is to find new ecologically and environmentally safer alternative, for the control of pre-harvest fruit drop.

Materials and Methods

Experimental site

The survey was conducted to check the incidence of pathological fruit drop in Kinnow growing areas, Bathinda, Fazilka and Muktsar districts of the Punjab during 2019-2021. Laboratory experiments were conducted in the Department of Plant Pathology, University College of Agriculture, and GKU.

Prevalence of pathological fruit drop in Kinnow

The comprehensive surveys were conducted to record the incidence of pathological fruit drop in Kinnow growing areas, Bathinda, Fazilka and Muktsar districts of the Punjab. Six trees were selected from each location and observations were taken at fortnightly interval starting from June-March (2019-20) to June- March (2020-21). Fruits showing typical symptoms of yellowing, browning and rotting near stem-end or soft watery rot at stem as well as stylar end, were considered as infected. The percentage fruit drop incidence was calculated.

The per cent fruit drop incidence was calculated by using the following formula:

Number of dropped fruits

$$\text{Per cent fruit drop} = \frac{\text{no. of fruit dropped}}{\text{Number of fruits harvested}} \times 100$$

Collection of samples, Isolation and identification of pathogens

Kinnow Fruits with typical symptoms were collected from the orchards, during the months of August to December. Fruits Showing rot on both stylar and stem-end, Brown soft and pliable Firm dark brown rot and, mummified and remained attached to twigs, were collected. Major Preharvest pathogens were isolated from the Infected fruits were cut into small pieces and surface sterilized with sodium hypochlorite (0.4%) for 30 seconds followed by a dip in Tween-20 for 45 seconds and then washed with distilled water. These fruit bits were further transferred to Potato Dextrose Agar (PDA).

Single spore culture

A small bit of mycelium was taken from the fungal culture, and put into 10 ml of sterilized water. From this spore suspension, 1ml solution was taken and 9 ml of sterilized water was added to make five serial dilutions. The spore suspension which was obtained at the end after all dilutions was spreaded over the water agar in Petri dishes. The Petri dishes were observed under the microscope and single spore was marked with a pointed marker, on the surface of Petri dish. These marked agar areas were cut with sterilized scalper and transferred to PDA slants and incubated at 25 ± 1°C.

Identification of the pathogen

Three Petri dishes were observed for recording colony colour and texture. Size and shape of conidia were obtained by averaging the results of 100 conidia. Continuous re-isolations were carried out on PDA slants to maintain the pathogenicity of the cultures.

Slide Culture Technique

In a glass Petri plate, a wet filter paper was placed at the base with steel ring over it, that formed the platform for glass slide and this assembly was steam sterilized. A thin layer of PDA was cut into small squares and was aseptically placed on the glass slide.

The agar block was then point inoculated with fungal cultures and a sterile cover slip was placed over it gently. The complete assembly was incubated at 28±2° C for 24-30 hrs and thereafter examined under binocular microscope (OLYMPUS BX 60).

Pathogenicity test and effect of inoculum concentration on disease development

Pathogenicity test was proved by Koch's postulate. After isolation of pathogen cultures were purified and maintained. Healthy kinnow of the same size were washed after being surface sterilized with disinfectant rinsed with sterile distilled water. Kinnow fruits were inoculated with three concentrations 50µl 100µl, 200µl by placing spore suspension which were adjusted with a hemocytometer to obtain concentration of pathogen containing 10^4 conidia/ ml. Inoculated fruits will be kept in trays and incubated at 28±1°C at room temperature. Fruits were inoculated by the following methods of inoculation, pinpricked and uninjured**.** Inoculations were done with the help of micropipette, in uninjured and pinpricked fruits. Inoculated fruits were wrapped with moist cotton and incubated at 25+1°C. Observations were taken in terms of days taken for appearance of visual symptoms. Re-isolation of the pathogen was carried out.

Preparation of aqueous plant extracts

Plant extract to be prepared by drying the plant material in the oven about 42 °C for 72hours. Aqueous extracts were prepared by 100g from each of the dried, powered plant sample to be weighed and mixed in a1000ml distilled water. Then the solution was boiled, cooled and filtered through the muslin cloth followed by filtrations by the whattman no.1 filter paper and the filtrate will be kept under normal room temperature.

Agar well diffusion method

Agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts.

Similarly to the procedure used in disk-diffusion method, a hole with a diameter of 6 to 8 mm is punched aseptically with a sterile cork borer or a tip, and a volume (20–100 µL) of the antimicrobial agent or extract solution at desired concentration is introduced into the well and placed for 2 hours. After that the agar plate surface is inoculated by spreading a volume of the microbial inoculum over the entire agar surface. Then, agar plates are incubated under suitable conditions depending upon the test microorganism. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested.

***In vitro* efficacy of BCAs against the Preharvest by MIC.**
Efficacy of *Trichoderma* spp, *pseudomonas* spp, *bacillus subtils* will be tested at different concentrations to be added separately in Potato Dextrose Agar against mycelial growth of *C. gloeosporioides* and *D. natalensis*, *A.citri*, using well diffusion method (Hasani *et al.*, 2014). *T.harizanum* use in three different concentrations *viz*, 250ppm, 500ppm and 750ppm. 100µl of spore suspension $1x10^8$/ml cfu (*B. subtils)* and 2%, 4%, spore suspension$1x10^8$/ml cfu (*P. inflorescence)* were used and efficacy evaluated by well diffusion method to study their effect on growth of Preharvest pathogens. 100µl of suspension each concentration were used to fill the well. The Petri dishes without BCAs, plant extract and fungicides were served as control.

***In vitro* efficacy of plant extract against the Preharvest pathogens**
The Two plants extracts i.e *Allium sativa* and *Azadirachta indica* were evaluated against mycelial growth of *C. gloeosporioides* and *D. natalensis*, *A.citri* using well diffusion method. (Hasani., *et al.* 2014) under *in vitro* condition, three different concentrations *viz*, 250ppm, 500ppm and 750ppm of *A.sativa* were used in well diffusion method. 2%, 4% and 6% concentrations of neem extract were evaluated by filling 100µl suspension in well diffusion method to study their effect on growth of Preharvest test pathogens. The Petri dishes without the of plant extract smearing, served as control.

***In vitro* efficacy of fungicides against the Preharvest pathogens:** Carbendazim and mancozeb at concentration, 250, 500 and 750 ppm each were evaluated by well diffusion method against mycelial growth of *C. gloeosporioides* and *D. natalensis*, *A. citri* using well diffusion method. (Hasani., *et al.* 2014) to study their effect on growth of Preharvest test pathogens.100µl of suspension each concentration were used to fill the well. The Petri dishes without fungicides smearing, served as control.

Statistical Analysis
Results obtained were reported as mean ± SD of triplicate measurements. Significance differences for multiple comparisons were determined by One-way ANOVA followed by Duncan test with P = 0.05 using SPSS (version 19).

Results and Discussion
Prevalence of pathological fruit drop in Kinnow mandarin at different locations
Prevalence of pathological fruit drop in Kinnow mandarin was recorded at Bathinda, Muktsar and Fazilka. The data represented in Fig. 1 indicates that maximum fruit drop in Kinnow plantation was recorded at Bathinda (46%), followed by Muktsar (40%) and Fazilka (32%). Significant difference was observed in fruit drop incidence during each month at all the selected locations. Fruit drop incidence was maximum from September to December. Maximum fruit drop incidence was observed from 15th to 30th October (2019). Similar trend was observed during 2020 at all locations of Punjab region.
Data presented in Fig 2. Shows that at all the locations fruit drop started from June and showed increasing trend till second fortnight of October, thereafter it showed declining trend up to second fortnight of January. Highest fruit drop was recorded from 30th September 2019 to 30th October 2019. Period from second fortnight of August to first fortnight of November is very crucial as maximum fruit drop takes place during these months. Fruit drop was significantly higher during September-October at all the locations. Similar trend was observed during 30th September to 30th October 2020 and data represented in Fig. 3. According to (Peres *et al.*, 2002) [30] inoculum availability and relative humidity the main basic factors which influenced the post bloom fruit dropping in citrus. Prolonged damp weather has been proved favorable to pathological fruit dropping in citrus (Singh, 2000; Agrios, 2005) [35, 2].
According to an experiment conducted by (Arora *et al.*, 2008) [3] and (Nawaz *et al.*, 2008) fruit drop incidence was recorded to be 29.80 per cent and 49.03. Per cent, respectively. Kumar *et al.*, (2012) [20] reported that incidence of pathological fruit drop in Kinnow mandarin varies 45 to 70 per cent. Pathological fruit drop caused by *C. gloeosporioides* is most detrimental in the month of September and October (Thind *et al.*, 2011) [37].

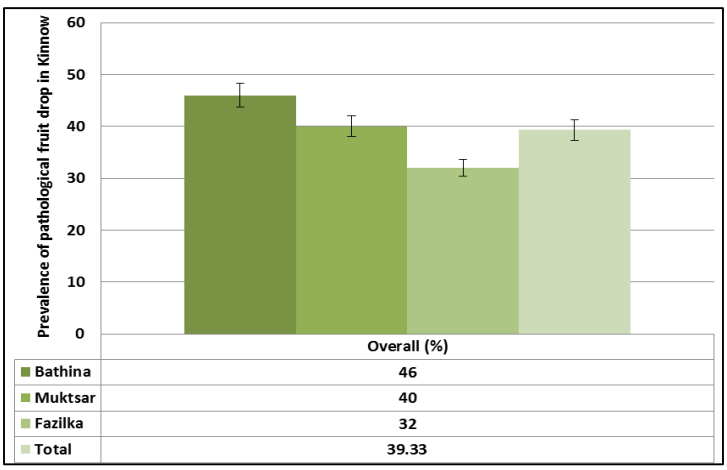

Fig 1: Prevalence of pathological fruit drop in Kinnow mandarin at different locations

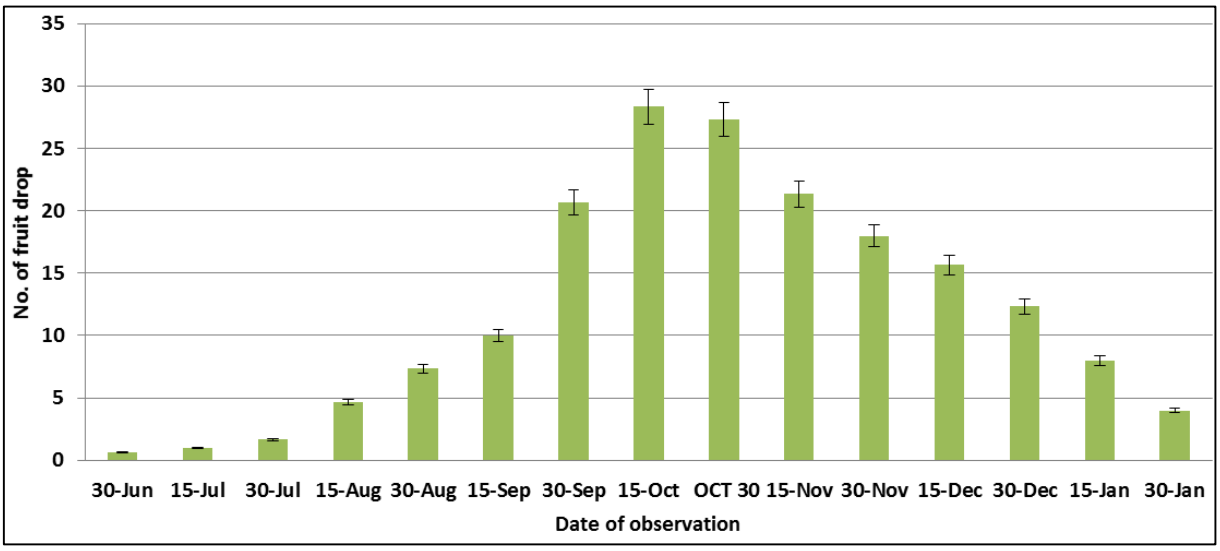

Fig 2: Periodical fruit drop in Kinnow mandarin at different locations in 2019- 20

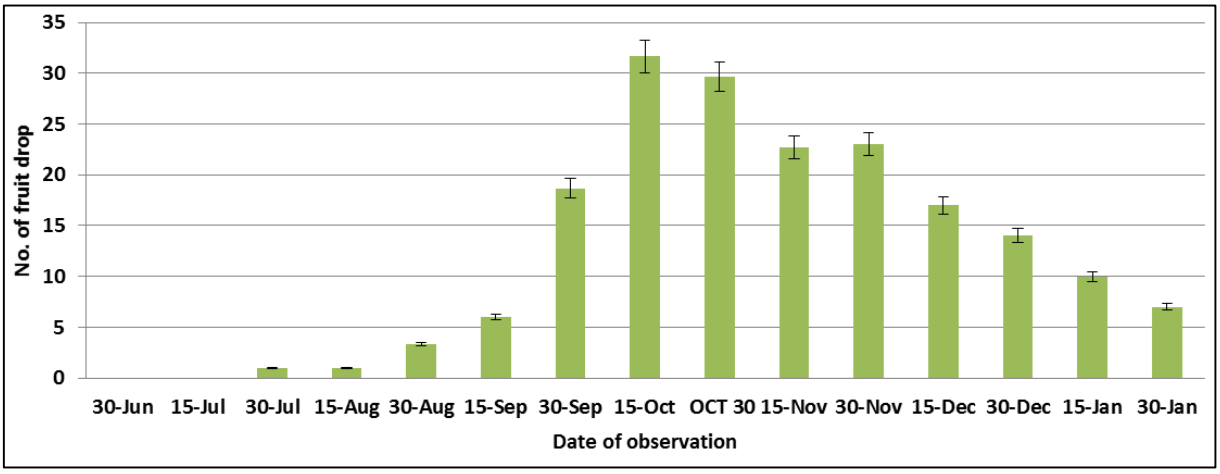

Fig 3: Periodical fruit drop in Kinnow mandarin at different locations in 2020-21.

Identification of *Colletotrichum gloeosporioides. D. natalensis. Alternaria citri*

The colony obtained from fruits showing brown discoloration at stem-end, on Potato Dextrose Agar (PDA) was white to dull white with smooth margins (Fig. 4). The mycelium was hyaline, superficial and septate. Acervuli started appearing in culture in the form of small black dots after 7 days.

Culture obtained on PDA media was white at first and subsequently became grayish white to gray and pink to reddish brown. Similar result were found by (Hanna *et al*., 2017; Alam *at al.* 2016) [11, 26] who studied that the color of *C. gloeosporioides* varied from white to grey and growth pattern was either circular with the mycelia showing a uniform growth pattern and radial in a ring like pattern. In this study, based on cultural and morphological identification the isolate was identified as *Colletotrichum gloeosporioides.*

Another fungus obtained from tissue of infected Kinnow fruits showing rotting both at stem-end as well as on stylar-end, appeared fluffy white initially and then colour changed to olive green (Fig. 5). Pycnidia were 128.25 - 256.75 µm in diameter and contained both hyaline and dark brown conidia. Wet (2009) [39] reported that conidia of *Diplodia* spp. are ellipsoidal to ovoid, they are initially hyaline and aseptate but become pigmented with age and sometimes septate. Brown colour Chlamydospores were also produced and present in chains. Thus based on these morphological characters the pathogen was identified as *D. natalensis*.

The colony of cultures obtained from fruits showing black rot, on Potato Dextrose Agar (PDA) was green olive to white at margin Colonies are fast growing, and are suede-like to floccose. Microscopically, branched acropetal chains (blastocatenate) of multicellular conidia (dictyoconidia) are produced sympodially from simple, sometimes branched, short or elongate conidiophores. Conidia are obclavate sometimes ovoid or ellipsoidal, often with a short conical or cylindrical beak, pale brown, smooth-walled or verrucose dark green to black with a white margin after 3 days of incubation (Fig.6). Based on these morphological characteristics and the description of *Alternaria* (Woudenberg *et al*. 2013; Alam *at al.* 2016) [40, 26], thus based on these morphological characters the pathogen was identified as. *Alternaria citri.*

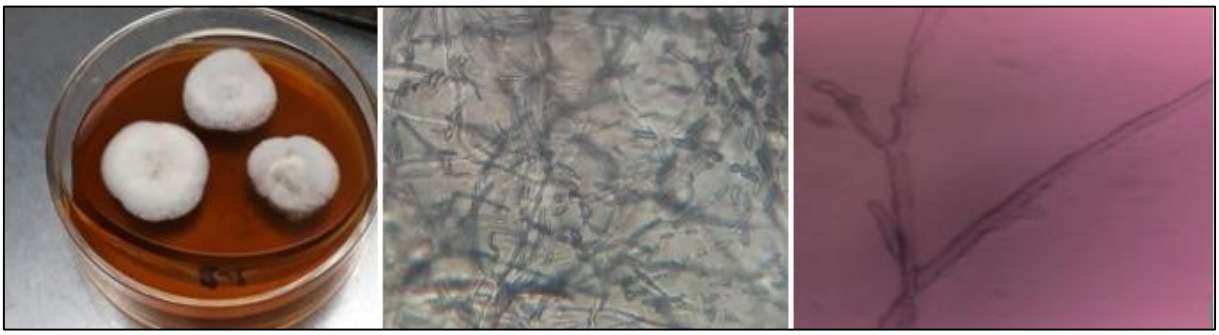

Fig 4: *C. gloeosporioides;* (a) Culture (b) Conidia (c) mycelium

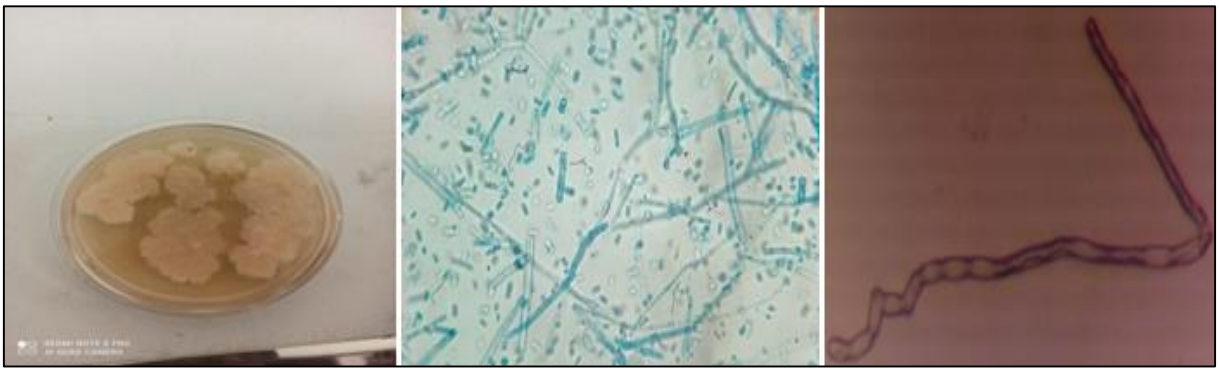

Fig 5: *D. natalensis;* (a) Culture (b) conidia (c) Chlamydospore

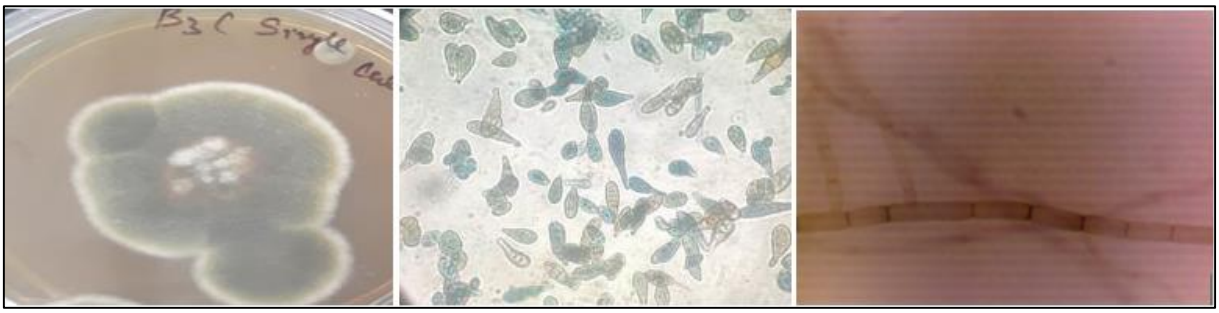

Fig 6: *A. citri;* (a) Culture (b) spores (c) Conidiophores

Pathogenicity tests

2×10^4 spores/ml suspensions of *C. gloeosporioides induced Symptoms* in pinpricked kinnow on 6th day as fruits developed a brown watery rot which started from stem as well as stylar end whereas in uninjured surface inoculated with same quantity symptoms developed within 8th day. Symptoms appeared in pinpricked kinnow inoculated with 2×10^4 spores/ml suspension of *D. natalensis* within 3rd days as fruits developed a brown watery rot which started from stem as well as stylar end whereas in uninjured surface inoculated with same quantity symptoms developed within 5th day (Fig.7). Kaur (2000) [19] inoculated Kinnow fruits by injecting 2.12 x 10^4 spores/ml of *D. natalensis*, using syringe and observed complete Rotting of fruits after 7 days. *A.citri* shows symptoms within 7th day as compared to uninjured inoculation in which symptoms appeared with in 9th day. In a study, it was found that 10^8 conidia/ ml^{-1} of *A. alternata* resulted in 93.4% infection recorded after 7 days of incubation. It was evident that 10^6 conidia ml-1 could serve as the inoculum potential of *A. alternata by* (Verma 2004).

Fig 7: (a) inoculum potential *D.natalenis* (b) inoculum potential *A.citri* (c) control

***In vitro* studies of biocontrol agents against pre harvest pathogens**

Effect of plant extract on formation of zone of inhibition against pre harvest pathogens Colletotrichum *gloeosporioides, Diplodia natalenis, Alternaria citri*

Maximum zone inhibition was brought out by *A.sativa* at 750ppm conc. 5mm, 16mm and 2mm for *C. gloeosporioides, A. citri and D. natalensis.* Studies have indicated that growth of pathogens was strongly inhibited under *in vitro* conditions by *A. sativa*. Our results are similar to those of lamsal *et al.* (2011) [22] who reported the effect of botanicals on growth of *A. citri* and proved that growth of *A. citri* was inhibited by garlic, neem, mint basal pat 60% conc. No zones of inhibition were recorded from the various conc. of *A.indica* against pre harvest pathogens. Our finding are confirmatory with ingole *et al.*, (2018) [24] who studied that 1% of neem oil were not inhibit the growth of *Colletotrichum gloeosporioides*, *Penicillium digitatum* and *Trichoderma viride, Geotrichum candidum* and *Aspergillus Niger* by paper disc method. Degradation of environment due to pesticide chemicals is a serious issue and they have contaminated almost every component of environment. Since, chemicals have harmful effects on the environment and use of botanical is the alternative approach for disease management that is eco-friendly and without any harmful impact on environment.

Effect of BCAs on formation of zone of inhibition against pre harvest pathogens *Colletotrichum gloeosporioides, Diplodia natalenis, Alternaria citri*

From results, revealed that *in vitro* conditions all BCAs inhibited the growth of all pathogen except *pseudomonas fluorescence* when compared to the control, among different BCAs maximum inhibition was brought out by *Bacillus subtilis* ($1x10^8$ /ml cfu). The diameter of zone inhibition was highest in case of *A. citri* (18mm).minimum zone of inhibition was found in case of *D. natalenis* (4mm). *T. Harzianum* performed superior than all tested BCAs against Preharvest pathogen. No distinct zone of inhibition was observed in case of *C. gloeosporioides* because the *T. harzianum* grow faster than the *C. gloeosporioides* which give an important advantages in the competition for space and nutrients with the pathogenic fungi (Barbos *et al*,. 2001) [5]. Highest zone of inhibition was recorded at 750ppm conc. in case of *D. natalenis* (22mm) followed by *A. citri* (6mm).

There are many studies that *Trichoderma* and *Bacillus* species showed strong antimicrobial activity against *A. alternata* (Abbo *et al*., 2014) [1]. Similarly, (Fuga *et al*., 2011) [10] evaluated that *in viro* conditions the mycelia growth of *C. gloeosporioides*, was inhibited by various soil bacteria.) The efficacy of *Trichoderma* in citrus disease management was also reported by others workers (Hanna *et al*., 2017; Harman *et al*., 2004) [11, 12].

Effect of fungicides on formation of zone of inhibition against pre harvest pathogens *Colletotrichum gloeosporioides, Diplodia natalenis, Alternaria citri*

The fungicides namely, Mancozeb and Carbendazim at three different concentrations *viz*., 250, 500 and 750 ppm and control were evaluated under laboratory conditions. Carbendazim and mancozeb were give significantly superior result. Minimum mycelial growth was recorded in Carbendazim, while maximum mycelial growth found in Mancozeb. Complete zone of inhibition was recorded in Carbendazim against *C. gloeosporioides* at all concentrations. Overall mean of zone of inhibition by carbendazim 7mm) against *D. natalenis* and against *A.citri* (11mm), while in mancozeb treatment, overall mean of zone of inhibition against *C. gloeosporioides* (6.66mm), against *D. natalenis* (6.33mm) and against *A.citri* (9mm). The efficacy of both fungicides in citrus disease management was also reported by others workers (Kaur *et al.*, 2004; Filoda 2008 & chand *et al*., 2013; Azad *et al*., 2014) [18, 9, 6, 4].

Conclusion

Pathological pre-harvest fruit drop was maximum from September to December causing reduction in yield and quality of harvested produce. Therefore, it is imperative to adopt integrated management approaches to effectively manage fruit drop in a sustainable way. Microbial and botanicals have the potential to control the Preharvest pathogens in Kinnow mandarin. Mycelial growth was clearly affected by the uses of biocontrol agents and botanical (garlic) against Preharvest pathogens in Kinnow such as *Colletotrichum gloeosporioides, Diplodia natalenis and Alternaria citri*. Thus, it can be concluded that integrated plant disease management (IPDM) is an ecologically and economically viable option to enhance the crop quantity and quality.

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