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Management of blue mould of Mandarin

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Abstract
Mandarin belonging to the family Rutaceae, are native to the tropical, sub- tropical regions of Asia. Blue mould of mandarin is one of the important post-harvest diseases caused by *Penicillium expansum*. Under *in vitro* conditions, GRAS salts (sodium carbonate, sodium bicarbonate, copper sulphate, Ammonium carbonate, Potassium carbonate, Sodium salicylate, Sodium sulfite) were evaluated against *Penicillium expansum*. Among GRAS salts tested Ammonium carbonate 15mM, 20mM and 25mM recorded maximum percent inhibition of 95.24, 98.68% and 96.90% respectively, followed by Sodium carbonate 15mM, 20mM and 25mM recorded percent inhibition of 85.41, 90.97 and 94.82% respectively. GRAS salts found effective under *in vitro* were evaluated under *in vivo* conditions. *In vivo* results revealed that Ammonium carbonate 25mM was efficient with 59.25 disease incidence and 66.07 disease severity which was followed by Ammonium carbonate 20mM with 62.02 disease incidence and 67.61 disease severity.

Keywords: *Penicillium expansum*, mandarin, GRAS salts, Blue mould

Introduction

Mandarin (*Citrus reticulata*), belonging to the family Rutaceae, are native to the tropical, sub-tropical regions of Asia such as southern Asia and the Philippines. Fruits vary in size, color according to their variety and some are seedless. Mandarin trees are woody shrubs which attains height of 3.6 to 4.5m, sometimes up to 8m. When compared to the fruit, tree is more drought tolerant. Fruits are globose to oblate in shape, small in size which measures about 4–8 centimeters (Morton, 1987) [1] color of the fruit is orange, orange-yellow, or orange-red (Karp, 2016) [2]. The most distinctive feature of mandarin orange is easy-to-peel skin, i.e. easy to remove the peel so it is called loose-skin orange.

Mandarin is a very good source of vitamin C, it also contains other vitamins like vitamin A, Thiamine (B_1), Riboflavin (B_2), Niacin (B_3), Pantothenic acid (B_5), vitamin B_6, Folate (B_9), Choline, and vitamin E. In addition, mandarin also contains some of the important minerals like calcium, iron, magnesium, manganese, phosphorus, potassium, sodium, zinc, being a plant product there is no cholesterol and fat. In a 100-gram reference serving mandarin orange consists of 85% of water, 13% carbohydrates, 37mg calcium, 26.7 mg of vitamin C. Mandarins are helpful in prevention of many diseases like anaemia, cancer, hypertension and influenza, as mandarins are rich source of minerals it will prevent micronutrient deficiencies, obesity and problems related to over nutrition (Anon. 2019a) [3].

On the basis of world scenario, area under orange cultivation is 3.97mha, production 73.19mt and productivity of 18.46t/ha. Brazil stands first in orange production, with an area of 0.66mha, production 17.25mt and productivity of 26.18t/ha followed by china with an area of 0.51mha, production 8.55mt and productivity of 16.91t/ha. India stands third position in orange production (Anon. 2019b) [4].

During 2018-19, the area under orange cultivation in India was 428.3ha, production 5101.2mt and productivity of 11.9mt/ha, and out of total area under citrus cultivation mandarin orange occupies nearly forty percent. In India, states which cultivate mandarin fruits are Madhya Pradesh, Maharashtra, Punjab, Rajasthan, Assam, Sikkim, Meghalaya, Mizoram, Tripura, Arunachal Pradesh and Andhra Pradesh. Madhya Pradesh is the leading state in orange production, with an area of 121.11ha and production 2103.64mt. Maharashtra is the second leading state with an area of 107.32ha and production 797.95mt (Anon.2019b) [4].

In the north east region mandarin is one of the important fruit crops and from this region 17 species, 52 varieties, 7 hybrids of citrus have been discovered. Among states of North East, Assam is the leading state in production followed by Arunachal Pradesh, Nagaland, Meghalaya, Mizoram, Manipur, Tripura and Sikkim (Anon. 2019b) [4]. North east region is a

repository house of many citrus species, still these regions are not successful in commercial cultivation of citrus spp because of genetic erosion, shifting cultivation, traditional agriculture practices, scattered land holdings, high rainfall and high humidity conditions (Hazarika, 2012) [5]

In Nagaland, around 60% of the population is engaged in the agriculture sector which contributes 27.47% to the state income. Area under citrus/ orange cultivation in Nagaland is 6.52ha and production is 47.33mt (Anon. 2019b) [4]. Where climate of citrus growing areas is humid subtropical with a mean rainfall of 2000mm. The maximum and minimum temperatures vary from 32˚C and 22.5˚C respectively with 99-70% relative humidity.

Physiologic, pathogenic factors influence post-harvest quality of citrus fruits and because of high water content, low pH i.e. acidic in nature pH range 2.2–4 it leads to majority of the decay by bacteria, fungi, viruses. 10 to 30 percent of losses are due to fungal diseases (Agrios, 2005) [6]. Some of the postharvest diseases caused by fungi are black rot (*Aspergillus niger*), blue mould rot (*Penicillium italicum*), soft rot (*Rhizopus stolonifer*), pink rot (*Trichonem aroseum*) and brown rot (*Botrydiplodia theobromae),* green mould rot (*Penicillium digitatum*) (Rao and Vaidya, 1995) [7].

Quality and shelf life of citrus fruits are influenced by mineral composition, but mineral composition of fruits is influenced by orchard type, location of field, climate and soil types (Nunes, *et al*., 2010; Munoz, *et al*., 2011) [8, 9]. Postharvest losses of fruits and vegetables varies according to the species, methods of harvesting, methods of transportation, and storage facilities. After harvest fruits and vegetables will lose resistance, and because of high water and nutrient content leads to attack of bacteria and fungi (Droby *et al.,* 1992) [10]. Optimum temperature, moisture and maturity stage of fruits are suitable conditions for the growth of microorganism (Aidoo, 1991).

Penicillium spp causes majority of losses, Link in 1809 created genus *Penicillium*, '*Penicillus*'- which means brush like sporulating structures (Nunes *et al*., 2010) [8]. The *Penicillium* genus consists of more than 150 species but only few of them are important pathogens (Samson and Pitt, 2000) [12]. Blue mould rot (*Penicillium italicum*), green mould rot (*Penicillium digitatum*) are most significant, whereas *P. expansum* has a wide range of host which infect apples, pear, and peaches. There are evidences which indicate *P. expansum* causing fruit rot of mandarin (Moosa *et al*., 2018) [13].

Many citrus species and varieties are attacked by phytopathogens after harvesting, leading to high losses in terms of value and volume. To minimize these losses, chemical fungicides which are traditional, cheap and effective have been used. However, due to continuous use of fungicides it leads to fungicide resistance amongst the fungal pathogens and residues of toxic chemicals on the fruit surface leading to health issues. Hence use of alternative methods like Generally regarded safe chemicals (GRAS) which are safer and ecofriendly are gaining much importance.

In the past few years, several experiments have proved the antifungal properties of GRAS salts (Askarne *et al.,* 2013; El-Sayeed *et al*., 2013) [17, 16]. GRAS salts are gaining importance as a promising alternative measure because of antifungal property, non-phytotoxicity, biodegradable and which are safer for public health and eco-friendly in nature, and by use of these salts, there is a low chance of development of resistance among postharvest pathogen.

As the percentage of postharvest losses of mandarin are high, it is necessary to study effective control measure which can minimize these losses. Therefore, the present study was designed to evaluate the efficient GRAS salts for the control of *Penicillium* spp.

The objectives of the present work are:

1. To isolate and identify the pathogen.
2. To evaluate suitable GRAS salts for management of blue mould in *in-vitro.*
3. *In-vivo* evaluating of GRAS salts (Generally Regarded as safe chemicals), in management of blue mould.

Materials and Methods

The present studies on "Management of blue mould of mandarin" were carried out in the Department of Plant Pathology, Nagaland University, Medziphema Campus, School of Agriculture Sciences and Rural Development, Medziphema, Nagaland. Details of materials used and methods followed are presented below.

General information

Location

The campus is situated approximately at 25˚45'43'' North latitude and 93˚53'04'' East latitude at an elevation of 310 m above mean sea level.

Inoculum used

Source of the pathogen

From the local market, infected mandarin fruits which showed characteristic symptoms were collected and brought to the laboratory for isolation and identification of the pathogen. Collection of infected fruits was done in a sterile bag to prevent contamination by other pathogens.

Isolation of the pathogen

Infected mandarin fruits which showed characteristic symptoms were brought to the laboratory, fruits were cleaned by using the tap water, allowed for drying. Later small portions of the infected tissue were taken from the margin of diseased and health portion on the fruit surface, surface sterilization of infected tissue was done with 1:1000 mercuric chloride, followed by three washes with sterile water, infected tissue was dried by using sterile blotting paper. Petri plates containing PDA were inoculated with the infected tissue under aseptic conditions and these Petri plates were incubated in BOD at 27 ± 1 ˚C and examined details of mycelial growth. By using hyphal tip culture pure culture of the pathogen was obtained (Sinclair and Dhingra, 1995) [18] and was further cultured on PDA slants so the slants of the culture were preserved in the refrigerator (4˚C) for further studies.

Identification of the pathogen

The morphological characters of the pathogen were studied on the host by inoculating the isolated test pathogen on healthy fruits. With the help of pin prick method described below as well as in the cultures growing in PDA the pathogen was identified. Based on morphological, cultural characters and along with the reports of ITCC (Indian Type Culture Collection) Division of Plant Pathology, IARI, New Delhi, pathogen was identified as *Pencillium expansum*

Pin prick method

Pin prick method was used for inoculation of the causal organs for the experiments. In this method sterilized needles were taken for making injury on the surface of the fruit.

Conidial suspension of 10^6/ml in sterilized distilled water was prepared by taking seven days old culture of the fungus in Petri plates. Later sterilized pins were dipped in the conidial suspension and the fruits were inoculated by piercing the skin of the fruits up to 1 mm depth by sterilized pins.

Pathogenicity test

The pathogenicity test of the causal agent (*P. expansum*) was done with the help of Koch's postulates under laboratory conditions by inoculating healthy fruits. Surface sterilization of the fruits were done by using 1% Sodium hypochlorite, followed by three washes with sterile water later fruits were allowed for air drying. Fruits were injured 1 mm depth by using sterile needle, followed by inoculation of fruits by 10^6 conidial suspension, in case of control, fruits were inoculated by distilled water. Fruits were maintained at room temperature and observations were recorded on occurrence of typical mould rot on the fruits. The test pathogen was re-isolated on PDA medium from diseased fruits, inoculated on healthy fruits and observations on production of symptoms were recorded to prove the pathogenicity of the fungus as given in koch's postulate.

Maintenance of pure cultures

By using hyphal tip method, culture of the pathogen was maintained in the slants containing the PDA for further study

Layout of the experiment

***In vitro* test of Gras Salts**

By using poison food technique (Sinclair and Dhingra, 1995) [18]. Different concentration of GRAS salts was incorporated into PDA individually in different conical flasks, each containing 100ml of sterile molten PDA. These were added to the PDA when it is cooled, later the flasks were gently shaken for 2 minutes to allow for a proper mixing with the medium. Then 20ml of the media was poured in to the Petri-plates (90mm diameter). Streptomycin was added to the medium at the time of pouring to prevent bacterial contamination. From seven-day old test culture a uniform disc of 0.5cm was cut with the help of cork borer and inoculated aseptically and placed on to the center of the Petri-plate. These plates containing PDA amended with different concentration of GRAS salts was kept upside down for better contact of pathogen to the media. The plates were then incubated at 27±1ºC in BOD. The colony diameter (cm) of the pathogen was determined by measuring the average radial growth on the 7th day after inoculation, when the control plates were full. Average radial growth was recorded by using a measuring scale from the lower view of the Petri-plates. Based on the observations recorded, percent inhibition of the test pathogen was calculated using the formulae given by Vincent (1927) [19].

Table 1: GRAS salt treatment against *Pencillium expansum* causing blue mould of mandarin

Gras Salt	Concentration-1	Concentration-2	Concentration-3
Sodium carbonate	15mM	20mM	25mM
Sodium bicarbonate	15mM	20mM	25mM
Ammonium carbonate	15mM	20mM	25mM
Copper sulfate	15mM	20mM	25mM
Potassium carbonate	15mM	20mM	25mM
Sodium salicylate	15mM	20mM	25mM
Sodium sulfite	15mM	20mM	25mM

Treatments

T_0: Control
T_1: Sodium carbonate at 15mM
T_2: Sodium carbonate at 20mM
T_3: Sodium carbonate at 25mM
T_4: Sodium bicarbonate at 15mM
T_5: Sodium bicarbonate at 20mM
T_6: Sodium bicarbonate at 25mM
T_7: Ammonium carbonate at 15Mm
T_8: Ammonium carbonate at 20mM
T_9: Ammonium carbonate at 25mM
T_{10}: Copper sulfate at 15mM
T_{11}: Copper sulfate at 20mM
T_{12}: Copper sulfate at 25mM
$T_{13:}$ Potassium carbonate at 15mM
$T_{14:}$ Potassium carbonate at 20mM
$T_{15:}$ Potassium carbonate at 25mM
T_{16}: Sodium salicylate at 15mM
T_{17}: Sodium salicylate at 20mM
T_{18}: Sodium salicylate at 25mM
T_{19}: Sodium sulfite at 15mM
T_{20}: Sodium sulfite at 20mM
T_{21}: Sodium sulfite at 25mM

***In-vivo* experiment**

Source of Fruit

Citrus fruits which were healthy without any physical injuries, wounds and without any postharvest treatments was collected directly from the farmers field of Wokha district, Nagaland.

***In vivo* evaluation of GRAS Salts**

GRAS Salt, Ammonium carbonate at 15Mm, 20mM and

25mM and Sodium carbonate at 15Mm, 20mM and 25mM found effective under *in vitro* conditions were further evaluated for *in-vivo* trails on the fruit. Healthy citrus fruits without any physical injuries, wounds were surface sterilized with 1% Sodium hypo chlorite, followed by three washes with sterilized water and fruits were air dried. Fruits were injured with sterile needle and inoculated with 10^6 conidial suspension of the test pathogen, after 2h fruits were dipped in the GRAS salt solution for 2-3min. In case of control, fruits were dipped in sterile water. After inoculation fruits were maintained at room temperature. Observations were recorded after 2 weeks of inoculation.

Treatments

T_0: Control
T_1: Sodium carbonate at 15mM
T_2: Sodium carbonate at 20mM
T_3: Sodium carbonate at 25mM
T_4: Ammonium carbonate at 15mM
T_5: Ammonium carbonate at 20mM
T_6: Ammonium carbonate at 25mM

Observations recorded

Percent inhibition of the test pathogen (Vincent, 1927) [19].

PI = C-T/C× 100

Where,
PI = percent inhibition of the pathogen
T = Growth of the pathogen in treatment
C = Growth of the pathogen in control

Disease incidence

Disease incidence (%) = [(number of rotten wounds / number of total wounds)]x100

Disease severity

$$\text{Disease severity (\%)} = \frac{[(\text{average lesion diameter of treatment/average lesion diameter of control})]}{(\text{Askarne } et\ al.,\ 2013)} \text{x100}$$

Statistical Analysis

In the present experiment Completely Randomized Design (CRD) was used for statistical analysis. To determine whether the collected data was significant or non- significant, the calculated value 'F' was compared to F- table at 5% probability level. When the treatment was significant Standard error of mean and Critical difference was calculated.
CD0.05= 1.414 x S.Em x t 0.05 for error degrees of freedom
The standard error of mean (S.Em) was calculated by using the formula:
S.Em± =√error mean square/replication
Where S.Em = standard error mean.
T0.05 = Table value of students 't' obtained at 5% level of significant.

Results and Discussion

The present experiment was carried out to study Management of Blue Mould of Mandarin. The results of all treatments were statistically analyzed to evaluate the efficacy of the GRAS salts applied. In this chapter results of all treatments are presented and explained with suitable tables, photographs, and figures.

Symptomatology

Pencillium expansum

P. expansum is one of the important pathogens which causes blue mould in mandarin during postharvest period and leads to severe economic losses. It has wide host range which can infect apples, many other fruits and vegetables. Symptoms of *P. expansum* initially appear as soft, light brown watery lesions on the surface of the fruit later, due to production of conidia turn blue-green in color. *P. expansum* produces a mycotoxin known as patulin which is a secondary metabolite leads to severe health issues for consumers.

Isolation and identification of the pathogen

As described in the materials and methods isolation of the pathogen was done from naturally infected fruit, by using hyphal tip culture technique. To ensure consistency, pathogen was re-isolated and purified. Based on cultural, morphological characters, nature of the symptoms and along with the reports of ITCC (Indian Type Culture Collection) Division of Plant Pathology, IARI, New Delhi, pathogen was identified.

Cultural and morphological characters of the pathogen

Based on cultural, morphological characters and along with the reports of ITCC (Indian Type Culture Collection) Division of Plant Pathology, IARI, New Delhi, the isolated fungus (*P. expansum)* was identified. Mycelia of the *P. Expansum* were white with abundant conidia, giving the colonies of *P. expansum* blue green color. Colony margin of *P. expansum* was entire and narrow. *Penicilli* were ter-verticillate with septate stipes. Conidia were smooth, globose to sub-globose in shape (Vico *et al.* 2014) [20].

***In vitro* test**

Efficacy of GRAS salts against *Penicillium expansum* under *in vitro* conditions.

By using poisoned food technique, efficacy of GRAS salts *viz*., sodium carbonate, sodium bicarbonate, ammonium carbonate, copper sulfate, Potassium carbonate, Sodium salicylate, Sodium sulfite was evaluated at different concentrations against *P. expansum.*
The data presented in Table 2, shows that when compared to the control, all seven salts significantly inhibited the test pathogen. The maximum percent inhibition of pathogen was recorded with ammonium carbonate (98.68) when applied at 20mM which is followed by ammonium carbonate (96.90) when applied at 25mM. The least percent inhibition of pathogen was recorded with sodium bicarbonate (33.52) at 15mM. The highest mean percent inhibition of pathogen was recorded with ammonium carbonate (96.94) and the least mean percent inhibition of pathogen was recorded with sodium bicarbonate (34.91). Whereas, with respect to concentration, irrespective of the different chemicals applied, 25mM recorded the highest percent inhibition of pathogen (66.32) and least mean percent inhibition of pathogen (61.65) was recorded with 15mM.
However, statistical analysis revealed that different concentration of the chemicals *viz.* 15, 20 and 25mM had

significant effect on percent inhibition of pathogen. Treatments with different salts and interactions between treatments applied and concentration of the salts were found to have significant effect on percent inhibition of pathogen. Ammonium carbonate at 15Mm, 20mM and 25mM and Sodium carbonate at 15Mm, 20mM and 25mM were further evaluated under *in vivo* conditions.

Table 2: Per cent inhibition of *Penicillium expansum* in presence of GRAS salts under *in vitro* conditions.

GRAS salt	15mM	20mM	25mM	Treatment Mean
Sodium carbonate	84.41	90.97	94.82	89.40
Sodium bicarbonate	32.52	34.18	37.03	33.91
Ammonium carbonate	95.24	98.68	96.90	96.94
Copper sulfate	70.04	73.85	74.99	72.29
Potassium carbonate	67.23.	69.20	70.23	68.22
Sodium salicylate	52.36	53.96	54.69	53.67
Sodium sulfite	48.20	47.23	45.00	46.81
Control	0.00	0.00	0.00	0.00
Concentration mean	61.65	65.63	66.32	

Effect	CD(p=0.05)
Salt	0.60
Concentration	0.52
Salt x Concentration	1.03

The results obtained demonstrate that several food additives can inhibit significantly the growth of *P. expansum.* In our study, we found that ammonium carbonate gave maximum percent inhibition of mycelial growth of *P. expansum*. Several previous findings signified that ammonium carbonate has completely inhibited the *in vitro* mycelial growth of *Monilinia fructicola* by 100% (Palou, 2018) [21], Similarly Sivakumar *et al*. (2002) [23] reported that ammonium carbonate inhibited 100% of the growth of *Colletotrichum gloeosporioides*. Turkkan, (2014) [24] also reported that ammonium carbonate inhibited the growth of *Ilyonectria* causing root rot of kiwifruit by 100%. The effectiveness of ammonium carbonate against *P. expansum* might be due to reduction of turgor pressure which results in the collapse and shrinkage of hyphae leads to subsequent inhibition of sporulation of the pathogen (Sivakumar *et al.* 2002) [23].

Common food additives which are low-toxic chemicals and Generally Regarded As Safe (GRAS) compounds, have been evaluated against citrus pathogens (Talibi 2014) [25]. Palou *et al.* (2008) [26] and Smilanick *et al.* (1999) [22, 27] reported that sodium bicarbonate and sodium carbonate salts are effective in inhibition of green and blue moulds. Askarane *et al.* (2013) [17] reported that copper sulphate was efficient against green and blue mould.

Evaluation of GRAS salts under *in vivo* conditions

Based on the *in vitro* studies, GRAS salts found effective were further evaluated under *in vivo* conditions. Observations like disease incidence and disease severity were recorded after two weeks of inoculation (14th day)

From the data provided in Table 3 it was observed that the least incidence of the disease was recorded to be 59.25% in ammonium carbonate at 25mmM which was followed by ammonium carbonate at 20mmM with 62.02% and least effective with highest disease incidence of 86.35% was recorded from sodium carbonate (15Mm) Similarly, disease severity was found to be 66.07% in ammonium carbonate at 25mmM which was followed by by ammonium carbonate at 20mmM with 67.61% and least effective with highest disease severity is sodium carbonate at 15mM (88.79%).

Table 3: Evaluation of effective fungicides, essential oils and GRAS salts under *in vivo* conditions

Treatment	Concentration	Disease incidence	Disease severity	Mean
Control		100.00 *(90.00)	100.00 (90.00)	100 (90.00)
Sodium carbonate	15mM	86.35	88.79	87.57
Sodium carbonate	20mM	80.32	82.96	81.64
Sodium carbonate	25mM	75.56	78.23	76.895
Ammonium carbonate	15mM	65.23	68.36	66.795
Ammonium carbonate	20mM	62.02	67.61	64.81
Ammonium carbonate	25mM	59.25	66.07	62.83
CD(p=0.05)		0.92	1.74	

Perusal of the results revealed that ammonium carbonate at 25Mm was found as most effective in inhibition of pathogen and was found superior to all other treatments. that ammonium carbonate has completely inhibited the *in vitro* mycelial growth of *Monilinia fructicola* by 100% (Palou, 2018) [21], Similarly Sivakumar *et al*. (2002) [23] reported that ammonium carbonate inhibited 100% of the growth of *Colletotrichum gloeosporioides*.

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